ABSTRACT

Hericium erinaceus (Bull. Fr.) Pers. is a well-known culinary-medicinal mushroom that is used in traditional Chinese and Japanese cooking and herbal medicine to treat various human diseases. The objectives of the present investigation was to evaluate the gastroprotection and healing effects by aqueous extracts of the basidiocarps *H. erinaceus* against ethanol-induced gastric ulcers in rats. In acute toxicity study, all animals did not show any abnormal behavioural expressions and toxicological signs. Seven groups of rats were pre-treated orally with distilled water as the normal group; distilled water as ulcer group; 20 mg/kg of omeprazole as positive group; 50, 100, 200, and 400 mg/kg of extract as the experimental groups. One hour later, distilled water was given orally to the rats in normal group while ethanol was given orally to the ulcer control, positive control, and the experimental groups. The rats were sacrificed after an hour. Ulcer area, gastric wall mucus, gastric mucus barrier test, and histology and immunohistochemistry of the gastric wall were assessed. Gastric homogenates were analysed for Lipid peroxidation (LPO), superoxide dismutase (SOD), and catalase (CAT) content. The ulcer group exhibited significantly ($p < 0.05$) severe mucosal injury when compared to rats treated with omeprazole or extracts which showed significant ($p < 0.05$) protection against gastric mucosal injury. The mushroom extracts promoted protection as there was significant ($p < 0.05$) reduction of gross ulcer area. Further, the histological analysis showed marked reduction of edema and leucocytes infiltration in the submucosal layer when compared to the ulcer group. Immunohistochemistry studies showed up-regulation of HSP70 protein and down-regulation of Bax protein in rats pre-treated with extract. Significant ($p < 0.05$) increase in the mucus of gastric content and high levels SOD and CAT, reduced amount of LPO was observed in rats treated with mushroom extracts indicating the protective and / or healing effects of the mushroom

extracts. During the three days ulcer treatment test, *H. erinaceus* extract at a dose of 400mg/kg could significantly ($p < 0.05$) heal the ulcer that was induced by ethanol in the rats.

ABSTRAK

Hericium erinaceus (Bull. Fr.) Pers. merupakan cendawan yang boleh di makan dan amnya digunakan dalam perubatan tradisional cina dan jepun untuk mengubati pelbagai penyakit. Objektif kajian ini adalah untuk mengkaji kesan gastroproteksi dan pemulihan ekstrak akues terhadap ulser gastrik berpunca dari etanol. Dalam kajian acute ketoksikan, semua haiwan tidak menunjukkan tingkah laku yang luar biasa dan tanda-tanda keracunan.Tujuh kumpulan tikus diberi minum air suling sebagai kumpulan normal; air suling sebagai kumpulan ulser; 20mg/kg omeprazole sebagai kumpulan positif; dan 50, 100, 200 dan 400 mg/kg ekstrak sebagai kumpulan eksperimen. Satu jam kemudian, air suling diberi minum kepada tikus dalam kumpulan normal sementara etanol diberi minum kepada kumpulan kawalan ulser, kumpulan kawalan positif, dan kumpulan-kumpulan eksperimen. Tikus-tikus tersebut dikorbankan sejam kemudian. Kawasan ulser, mukus dinding gastrik, ujian pemisah mukus gastrik, dan histologi dan immunokimia dinding gastrik diperiksa. Homogenat gastrik dikesan untuk kandungan lipid peroxidasi (LPO), superoxide dismutase (SOD), dan catalase (CAT). Tikus- tikus dibawah kumpulan yang dijangkiti ulser menunjukan perubahan ketara ($p < 0.05$) dalam kecederaan mukosa yang parah berbanding dengan tikus-tikus yang dirawati dengan omeprazole atau kumpulan ekstrak yang menunjukkan sifat perlindungan terhadap kecederaan mukosa gastrik yang ketara ($p < 0.05$). Cendawan ini menunjukan kesan protektif terhadap ulser kerana ia menunjukkan pengurangan ketara kawasan ulser dan histologi menunjukkan pengurangan edema dan penyusupan leukosit lapisan submucosal berbanding dengan kumpulan ulcer. Immunohistokimia menunjukkan up-regulation protein HSP70 and down-regulation protein Bax dalam tikus-tikus yang telah dirawati dengan ekstrak. Peningkatan ketara ($p < 0.05$) dalam mukus kandungan gastrik dan paras tinggi SOD dan CAT, serta pengurangan jumlah LPO diperhatikan pada tikus

yang dirawat dengan ekstrak cendawan ini. Kajian terhadap ulser telah dijalankan selama tiga hari dan ekstrak *H. erinaceus* (400mg/kg) menunjukkan kebolehan untuk menyembuhkan ulser dalam kumpulan tikus yang telah diberikan etanol.

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LIST OF SYMBOLS AND ABBREVATIONS

m	: meter
mm	: millimeter
µm	: micrometer
nm	: nanometer
µl	: microliter
ml	: milliliter
g	: gram
kg	: kilogram
µg	: microgram
mg	: milligram
mg/ml	: milligram per milliliter
mg/kg	: milligram per kilogram
%	: percentage
°C	: degree Celsius
min	: minute
h	: hour
rpm	: rotation per minute
mol/L	: Molar
ANOVA	: analysis of variance
±	: plus-minus
w/v	: weight (of solute) per volume (of solvent)
M	: Molar
µM	: micro Molar
KUM	: kod Universiti Malaya
PBS	: phosphate buffer saline
$MgCl_2$	: magnesium chloride
TBA	: thiobarbituric acid
TCA	: trichloroacetic acid
TBARS	: thiobarbituric acid-reactive substances
H_2O_2	: hydrogen peroxide
S.E.M.	: standard error mean
>	: more than
<	: less than

1.0 Introduction

Peptic ulcers, including both gastric and duodenal ulcers, have been a major threat to the world's population over the past two centuries. In 2007, roughly 14.5 million people in the United States suffered from peptic ulcers, and this number had increased further to 14.99 million by year 2010. (Pleis *et al*., 2009; and Schiller *et al*., 2012). The two most common types of peptic ulcers are named after the location where the ulcer is found. Gastric ulcers are located in the stomach, whereas duodenal ulcers are found at the beginning of the small intestine or duodenum.

The predominant symptom of uncomplicated peptic ulcer is epigastric pain, which presents itself with other symptoms such as fullness, bloating, early satiety, and nausea. Generally, patients with duodenal ulcers suffer epigastric pain during the fasting state or even during the night, and this is usually relieved by food intake or acid-neutralising agents (Malfertheiner *et al*., 2009). Gastric ulcers are a serious gastrointestinal (GI) disorder, and occur when the gastric mucosa is impaired, leading to perforations of the stomach lining and then bleeding.

Generally, peptic ulcers result from an imbalance between increased aggressive factors in the body, such as acid and pepsin secretions (Wolfe & Sachs, 2002), and decreased defensive factors, such as mucus and bicarbonate secretions (Allen & Flemstrom, 2005), mucosal barriers (Peskar, 2001), mucosal blood flow (Abdel-Salam *et al*., 2001) and endogenous prostaglandin production (Peleg & Wicox, 2002). Some other factors which induce peptic ulcers include *Helicobacter pylori* infections (Peura, 1997), the ingestion of non-steroidal anti-inflammatory drugs (NSAID) (Pawlik *et al*., 2000), and psychological stress (Brogie, 1962). The production of free radicals, inhibition of cell proliferation, and infiltration of inflammatory cells are also factors involved in the pathogenesis of ulcers (Maity & Chattopadhyay, 2008). Many

ulcerogens produce excessive reactive oxygen species (ROS), which cause imbalances between aggressive and protective factors such as levels of cytokines, prostaglandins (PGs) and enzymes (COXs, NOS etc.), pro- and anti-angiogenic as well as tissue growth parameters.

The objectives of treatment for peptic ulcers are relief from pain, enhancement of healing and prevention of recurrence. Treatment for stomach and duodenal ulcers is decided by the doctor on the basis of a patient's age; overall health; medical history; extent of the pathogenesis; tolerance for medications, procedures or therapies; and expectations or preferences. Therapeutic strategies generally aim to restore balance between aggressive and defensive factors. In the past, the major therapies mainly aimed to reduce the secretion of gastric acids, which were considered as the main cause of ulcer formation. Nowadays, treatment strategy has changed to focusing on potentiating the mucosal defences along with reducting acid secretions (Wallace, 2005).

Anti-secretory agents as H_2-receptor antagonists and proton pump inhibitors and mucosal protective agents as sucralfate and prostaglandin analogs are very important drugs for the treatment of peptic ulcers. At the same time, each of these drugs can cause simpler to serious side effects like gynaecomastia that occurs with cimetidine (H_2-receptor antagonist) (Brogden *et al*., 1978) and enterochromaffin-like cell (ECL) hyperplasia that occurs in omeprazole (proton pump inhibitor) treatments (Ekman *et al*., 1985). Hence the interest of researchers in finding better and safer alternatives for the treatment of peptic ulcers and specifically in the gastroprotective effects of medicinal plants which may have fewer side effects.

Nowadays, natural products have not only gained a strong foothold in the pharmaceutical industry but have also inspired the search for new potential sources of bioactive molecules (Schmeda-Hirschmann & Yesilada, 2005). Herbs, medicinal plants,

spices, vegetables, and crude drug substances are considered potential resources in the fight against various diseases, including gastric ulcers. Some medicinal plants such as ginger (Yamahara *et al*., 1988), turmeric (Rafatullach *et al*., 1990) and aloe (Suvitayavat *et al*., 1997) are reported to have gastroprotective effects. Abdulla *et al*. (2008) reported that *Hericium erinaceus*, one of the well-known medicinal mushrooms, produced gastroprotective activity in rats.

Medicinal mushrooms have a history of applications in traditional oriental therapies. Mushroom-derived preparations continue to be used in modern clinical practice in countries such as Japan, China and Korea. Medicinal effects have been proved for many traditionally used mushrooms (Ooi & Liu, 2000), including extracts from species belonging to the genera *Auricularia*, *Flammulina, Ganoderma*, *Grifola, Hericium, Lentinula*, *Pleurotus, Trametes (Coriolus*), *Schizophyllum*, and *Tremella* (Wasser, 2002). Over the past twenty to thirty years, scientific and medical studies in Japan, China, Korea, and more recently in the United States, India and Malaysia, have increasingly demonstrated the potent and unique properties of mushroom-extracted compounds for the prevention and treatment of cancer.

Hericium erinaceus, a spine fungus which grows on the trunks, branches and stumps of trees, can be found throughout the northern hemisphere, and range from rare in central Europe to fairly common in southern England (Pegler, 2003). This mushroom not only tastes extremely good, but also several medicinal properties have been attributed to it. Previous studies have reported anti-tumor activities (Mizuno *et al*., 1995), antioxidant properties (Wong *et al*., 2007), antimicrobial effects (Wong *et al*., 2009a), the stimulation of neurite outgrowth in the cell line NG 108-15 (Wong *et al*., 2009b) and nerve regeneration (Wong *et al*., 2011).

Hericium erinaceus is a temperate mushroom that has been domesticated and is commercially grown in Malaysia. The extracts of this mushroom have both neurite-outgrowth stimulating effects (Wong *et al*., 2009b) and preliminary study had shown that gastric ulcer prevention effects of this mushroom (Abdulla *et al*., 2008). In the current study, extracts of tropical grown *H. erinaceus* were investigated to establish whether and how these have gastric ulcer prevention and healing effects.

The objectives of this study were to:

a) investigate the gastroprotective and ulcer healing properties of *H. erinaceus*.

b) measure the lipid peroxidation and antioxidant enzymes in gastric ulcers in a rat's stomach, induced by ethanol.

c) evaluate the effects of ethanol and *H. erinaceus* extract on HSP70 and Bax proteins in immunohistochemical staining.

2.0 Literature review

2.1 Gastric Ulcers

2.1.1 Anatomy of human stomach

The stomach is the most dilated part of the digestive tube, having a capacity of 1000–1500 ml in adults. It is located between the end of the oesophagus and the duodenum – the beginning of the small intestine. It lies in the epigastric, umbilical, and left hypochondrial regions of the abdomen, and occupies a recess bounded by the upper abdominal viscera, the anterior abdominal wall and the diaphragm. It has two openings and is described as having two borders, although in reality the external surface is continuous. The relationship of the stomach to the surrounding viscera is altered by the amount of the stomach contents, the stage that the digestive process has reached, the degree of development of the gastric musculature, and the condition of the adjacent intestines. However, borders are assigned by the attachment of the peritoneum via the greater and lesser omentum, thus dividing the stomach into an anterior and posterior surface (Daniels & Allum, 2005).

The main function of the stomach is to mix food with acid, mucus and pepsin and then release the resulting chyme, at a controlled rate, into the duodenum for the process of absorption. Gastric motility is controlled by both neural and hormonal signals. Nervous control originates from the enteric nervous system as well as the parasympathetic (predominantly vagus nerve) and sympathetic systems. A number of hormones have been shown to influence gastric motility: gastrin and cholecystokinin, for example, act to relax the proximal stomach and enhance contractions in the distal stomach. Other functions of the stomach include the secretion of intrinsic factors necessary for the absorption of vitamin B12 (Daniels & Allum, 2005).

The stomach wall is made of four layers: mucosa, submucosa, muscularis externa and serosa. The mucosa is the innermost layer, and is divided into a surface epithelium, a loose connective tissue called lamina propria, and a thin smooth muscle called muscularis interna or muscularis mucosae. The submucosa is the loose connective tissue, containing blood vessels and nerve endings. The muscularis externa is the muscular layer, consisting of an inner oblique muscle, a middle circular muscle and an outer longitudinal muscle. Finally, the serosa is the outermost layer (Marieb, 1998).

2.1.2 Rat stomachs

The rat's stomach comprises two regions: the non-glandular (forestomach) and glandular parts. The non-glandular stomach is lined by cornified squamous epithelium. The glandular stomach consists of the corpus, antrum and pyrolus, and is lined by a simple columnar epithelium. The non-glandular part is distinguished from the glandular part of the stomach by the limiting ridge, which prevents a reflux of gastric juice from the glandular part into the non-glandular part when the stomach is empty. The anatomy and physiology of a rat's stomach is not much different from the human stomach. There has been a considerable volume of research using the rat's stomach as tools to investigate the gastric function, so the results from rat models provide the basic information on humans as well (Sbarbati *et al*., 1995).

2.2 Peptic ulcer and gastric ulcer diseases

There are various kinds of ulcers, named after their respective locations in the digestive tract. Duodenal or peptic ulcers can be found in the intestines, while gastric ulcers are located inside the stomach. A gastric ulcer is considered as a necrotic lesion

penetrating through the entire mucosal thickness of the stomach. Peptic and duodenal ulcers are usually benign; however, gastric ulcers are more prone to becoming malignant. In this study, we focus on gastric ulcers induced by ethanol.

2.3 Factors that cause peptic ulcers

Peptic ulcers form because of an imbalance between increased aggresive factors, which breakdown the ability of the gastrointestinal mucosa to protect itself, and a decrease in the defensive factors which maintain mucosal integrity through endogenous defense mechanisms. These aggressive and defensive factors and their roles in peptic ulcers are described below:

2.3.1 Aggresive factors

2.3.1.1 *Helicobacter pylori* (*H. pylori*)

Helicobacter pylori is a spiral-shaped, pH-sensitive, gram-negative, microaerophilic bacterium that arises between the mucous layer and surface epithelial cells in the stomach. The ability of *H. pylori* to produce urease may be related to its pathogenic potential and survival in the stomach. Urease is an enzyme which can break down urea into carbon dioxide and ammonia. The ammonia is converted into ammonium by taking away a proton (H^+), leaving only hydroxyl ions. The hydroxyl ions then react with carbon dioxide, producing carbonate, which neutralizes gastric acid. Urease activity is low at neutral pH but can increase 10- to 20-fold as the external pH falls to between 6.5 and 5.5, and remains high at pH 2.5 (Scott *et al*., 1998). *Helicobacter pylori* also carry another protein – urel, which is a urea transporter that brings urea into the cytoplasm of the bacteria for urease to digest. *Helicobacter pylori*

cause gastric mucosal injury by direct effects, by changes in immune/inflammatory responses and by hypergastrinemia leading to increased acid secretions. *Helicobacter pylori* infections change inflammatory responses and damage epithelial cells directly by cell-mediated immune mechanisms or indirectly by activated neutrophils or macrophages attempting to phagocytose bacteria or bacterial products (Peura, 1997).

2.3.1.2 Nonsteroidal anti-inflammatory drugs (NSAID)

NSAIDs cause clinically significant ulceration, bleeding, or obstruction in 1–4% of patients chronically taking these drugs (Silverstein *et al*., 2000). NSAIDs inhibit cyclooxygenase (COX), the enzyme responsible for the conversion of arachidonic acid to prostaglandins (Vane, 1971). There are two isoforms of COX: COX-1 and COX-2 (Crofford *et al*., 2000). COX-1 is a ubiquitous constitutive isozyme producing prostaglandins responsible for maintaining gastrointestinal mucosal integrity, vascular homeostasis and renal functions (Crofford *et al*., 2000). COX-2 is a largely cytokine-induced iso-zyme producing prostaglandins that are associated with pain and inflammation (Crofford *et al*., 2000). NSAIDs inhibit both COX-1 and COX-2 to varying degrees (Gierse *et al*., 1995; and Gierse *et al*., 1999). Gastric injuries induced by NSAIDs are shown in Figure 2.1.

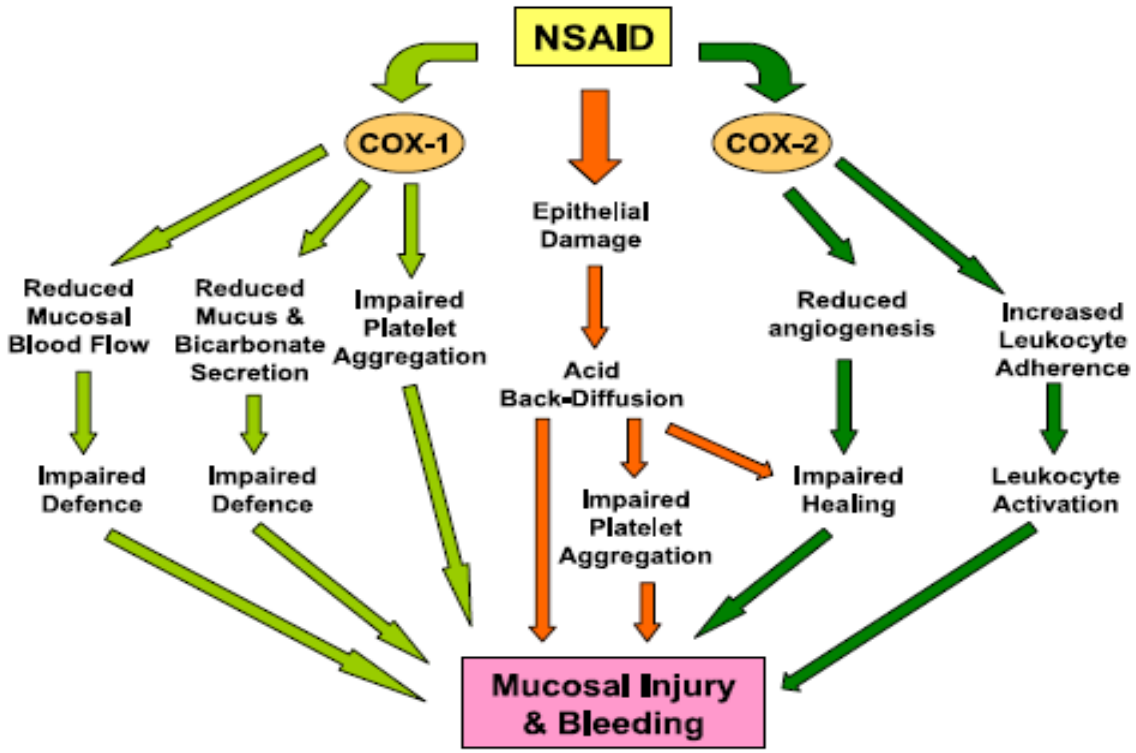

Figure 2.1: The pathogenesis of NSAID-induced gastric injury and bleeding. NSAIDs induce injury/bleeding via three key pathways: inhibition of cyclooxygenase (COX)-1 activity, inhibition of COX-2 activity, and direct cytotoxic effects on the epithelium. Effects produced via only one of these pathways (e.g. the selective inhibition of COX-1 and COX-2) are unlikely to produce significant damage (retrieved from Wallace, 2008).

Gana *et al*. (1987) reported that NSAIDs have the ability to reduce blood flow on the gastric mucosa. A reduction of gastric blood flow is usually not sufficient to result in significant mucosal injury. However, reduced mucosal blood flow can lead to the mucosa becoming more vulnerable to damage induced by luminal irritants, and damage the epithelial restitution process (Wallace & McKnight, 1990).

2.3.1.3 Stress

Psychological and physical stresses have both been shown to precipitate mucosal injury in animal models of peptic ulcer disease (Weiner, 1991). Stress ulcers are frequently seen in intensive care settings after surgery for various acute and severe diseases. The resulting pathophysiology interferes with blood flow and sporadic acid

secretions, and disrupts mucosal defences. Incubation, which is a common procedure during major surgery (especially cardiac, neural, or renal transplants), and major trauma, such as bum or spinal cord injury, increase the chances of patients getting peptic ulcer disease (Soll, 1989).

2.3.1.4 Smoking

Previous studies (Hull & Beale, 1985) have proved that cigarette smoking is an important risk factor which can cause peptic ulceration and delayed ulcer healing. However, the mechanism through which ulceration develops due to cigarette smoking still remains unclear.

2.3.2 Defensive factors

2.3.2.1 Mucosal defense

The term “mucosal defense” describes the various factors and components that permit the mucosa to remain intact despite frequent exposure to substances with a wide range of temperatures, pHs, and osmolarity, as well as to substances with detergent or cytotoxic actions, and bacterial products capable of causing local and systemic inflammatory reactions (Wallace & Granger**,** 1996). The gastric mucosa is not impermeable to damage by these agents, and mucosal injuries do occur reasonably regularly. However, the ability of the mucosa to repair itself rapidly can limit these to the most superficial layer of cells and prevent entry into the systemic circulation of substances detrimental to the organism. In a healthy organism, the resistance of the gastric mucosa to injury is increased when the stomach is irritated by certain factors, for

instance through an increment of mucosal blood flow and an efflux of mucus from surface epithelial cells.

2.3.2.2 The Epithelium

The epithelium is often described as the physical barrier of stomach. There are various components of mucosal defense on the luminal side of the epithelium, including gastric juice and mucus. Gastric juice has a number of constituents capable of decreasing bacterial colonization of the stomach, including acid, immunoglobulins, and lactoferrin. Giannella *et al*. (1973) demonstrated that acid is an important defensive factor, and that hypochlorhydria and achlorhydria can therefore increase the risks and exacerbate the severity of bacterial and certain parasitic infections.

The mucus that is secreted onto the surface of the stomach plays a number of roles: as a lubricant, to reduce physical damage to the epithelium by ingested materials, and as a trap for bacteria (Forstner, 1978; and Belley *et al*., 1999). Thus mucus can decrease the ability of bacteria to enter into the epithelium. Mucus creates an unstirred layer on the mucosal surface, which helps to maintain a near-neutral pH at that surface as well as acting as a physical barrier against luminal pepsin (Allen & Flemström, 2005). Schreiber and Scheid (1997) suggested that mucus retard the diffusion of protons, which can further help in maintaining a favourable pH at the apical surface of the epithelium.

Hills *et al.* (1983) reported that the surface of the stomach is hydrophobic, and therefore a barrier to acid back-diffusion, because of the presence of a surfactant-like layer of surface-active phospholipids. This layer is located either on the surface of the epithelium itself, or on the most luminal surface of mucus overlying the epithelium (Goddard *et al*., 1990). Aspirin or bile salts cause disruption in this layer, resulting in an

elevated diffusion of acid into the mucosa, and to mucosal necrosis (Goddard *et al*., 1990).

Although there are several "layers" of mucosal defense, experimentally reducing the effectiveness of the mucus-bicarbonate layer on the epithelial surface does not usually result in epithelial damage (Wallace, 1989). It is possible that may have an underlying ability to remain intact and functional when continuously exposed to high concentrations of acid. Sanders and colleagues (1985) reported that the apical membrane of cultured chief cells is highly resistant to damage by acid and that these cells can tolerate a solution of pH 2 for more than four hours without any damage to the cells. Takezono *et al*. (2004) revealed that gastric-surface mucus cells have a mechanism to resist the back-diffusion of acid into the mucosa that involves enhancement of the functional barrier.

2.3.2.3 Mucosal blood flow

Mucosal blood flow is triggered by sensory afferent neurons when irritants such as acid enter the subepithelial compartment, allowing the buffering of acid and the rapid removal of toxic substances, thus limiting the latter's penetration into deeper layers of the mucosa (Wallace & Granger**,** 1996). When the sensory afferents are stimulated, this causes a rapid increase in mucosal blood flow. Holzer and Sametz (1986) reported that excision of the sensory afferent neurons eliminates the "reactive hyperemic" response to topical irritants and thereby increases the chances of injury to the gastric mucosa. A constant delivery of plasma from the subepithelial blood vessels plays an important role in maintaining a repair-conducive microenvironment. An interruption in blood flow can result in a rapid decrease in pH at the site of the injury, leading to disruption of the repair process and damage progressing to deeper layers of the mucosa (Wallace & McKnight, 1990).

2.3.2.4 Ulcer healing

Healing a gastric ulcer requires restoring the epithelium, endothelium, connective tissue, and smooth muscle damaged during ulceration. Ulcer healing is a complex repair process that involves inflammation, cell proliferation (particularly at the ulcer margin), the formation of granulation tissue at the base of the ulcer, and angiogenesis (new blood vessel growth).

Normally, intestinal epithelial repair after injury involves different processes which can be considered partially independent (Figure 2.2). The initial, rapid response to damage involves the migration of surrounding epithelial cells to cover the denuded area (Argenzio *et al*., 1988; Lacy, 1988; and Feil *et al*., 1989) - a process called re-epithelialization. This process is crucial because a continuous epithelial "barrier" can protect granulation tissue from mechanical and chemical injury or infection. For small wounds, short restitution and proliferation phases may be sufficient to restore the monolayer. If the wound is large, immunologic responses and the deposition of protective granulation tissue may be necessary to restore epithelial continuity.

The re-epithelialization process involves epithelial cell migration and proliferation, and is activated and regulated by certain growth and transcription factors (Tarnawski, 2000; Vanwijck, 2001; and Chai *et al*., 2004).

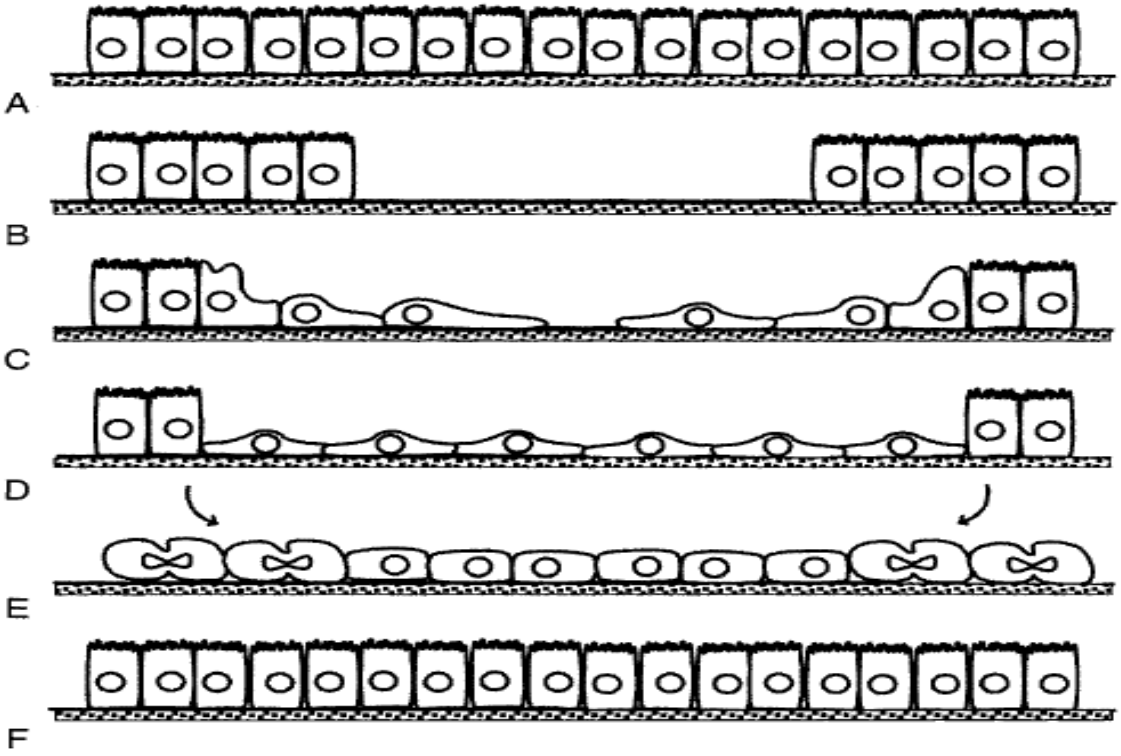

Figure 2.2: The basic processes of intestinal epithelial wound healing. When an undamaged mucosal surface (A) is subjected to an attack that strips away epithelial cells (B), the first response is loss of polarization of cells near the wound margin and conversion to a migratory phenotype (C). Over a process of minutes to hours, depending on the extent of the wound, cells flatten and move to cover the denuded area, re-establishing the protective barrier (D). Leader and follower cells often move as a unified sheet, maintaining rudimentary attachments during the restitution process. Cell proliferation restores the epithelial population (E), allowing cells to reform normal junctional complexes and retrieve a polarized columnar phenotype (F). (Frey & Polk, 2006)

Granulation tissue develops at the ulcer base two or three days after ulceration [(Tarnawski *et al*., 1990; Tarnawski *et al*., 1991; Tarnawski, (1993, 2000); and Cotran *et al*., 1999)]. Proliferating connective tissue cells such as macrophages, fibroblasts and proliferating endothelial cells found in granulation tissue form microvessels through the process of angiogenesis [(Tarnawski *et al*., 1990; Tarnawski *et al*., 1991; Tarnawski, (1993, 2000); and Cotran *et al*., 1999)]. Granulation tissue plays a crucial role in the ulcer healing process because it supplies connective tissue cells (a synthesizing extracellular matrix for restoring the lamina propria) and microvessels for the restoration of the microvasculature within an ulcer scar [(Tarnawski *et al*., 1991; Tarnawski, (1993, 2000); and Cotran *et al*., 1999)].

Angiogenesis is the process for forming a new microvascular network, which is considered essential for the healing of chronic gastroduodenal ulcers. Angiogenesis happens through a series of steps, which include: (a) degradation of the capillary basement membranes by matrix metalloproteinases; (b) endothelial cell migration into the perivascular space and proliferation; (c) the formation of microvascular tubes followed by anastomoses; (d) the establishment of lumina and basement membranes; and finally, (e) the formation of the capillary network (Tarnawski *et al*., 1991; Folkman & D'Amore, 1996; Risau, 1997; and Cotran *et al*., 1999). The formation of granulation tissue and generation of new microvessels through angiogenesis is induced by bFGF, VEGF, PDGF, angiopoietins, and other growth factors and cytokines, including IL-1 and tumor necrosis factor-alpha (TNF-α) (Folkman & D'Amore, 1996; Risau, 1997; and Tarnawski, 2000).

Extracellular matrix (ECM) is secreted by a wide range of cell types including fibroblasts, epithelial, smooth muscle and endothelial cells. It assembles into a network in the spaces surrounding cells which withdraws water and minerals and binds growth factors (Basson *et al*., 1993; Cotran *et al*., 1999; and Basson, 2001). ECM consists of fibrous structural proteins such as the collagens and elastins, adhesive glycoproteins including fibronectin and laminin and an amorphous gel composed of proteoglycans and hyaluronan. All the components above can form an interstitial matrix as well as the basement membrane (Basson *et al*., 1993; Cotran *et al*., 1999; and Basson, 2001).

The replacement of granulation tissue with a connective tissue scar changes the composition of the ECM. These growth factors not only stimulate the production of collagen and other connective tissue components but also modulate the synthesis and activity of metalloproteinases- enzymes which degrade these ECM components (Cotran *et al*., 1999). The end result of this process is a remodelling of the connective tissue, which is an important feature of ulcer healing (Cotran *et al*., 1999). Degradation of the

collagen and other ECM proteins is accomplished by the matrix metalloproteinases, enzymes dependent on zinc ions for their activity (Pai *et al*., 1998; Cotran *et al*., 1999; and Calabro *et al*., 2004). Some collagenases and their inhibitors are essential in the remodelling of connective tissue required for tissue defect repair and scar formation (Pai *et al*., 1998; and Calabro *et al*., 2004).

2.4 Medical treatment of gastric ulcer disease

Treatment of gastric ulcer disease usually involves a combination of antacids (aluminium hydroxide, magnesium hydroxide) (Preclik *et al*., 1989), H_2-receptor antagonists (e.g. cimetidine, famotidine, nizatidine, ranitidine) (Rainford, 1987), proton pump inhibitors (e.g. omeprazole, lansoprazole) (Sander, 1996), and complex bismuth salt (e.g. colloidal bismuth subcitrate (CBS)) (Tytgat, 1986). Unfortunately, patients have to take as many as 20 pills a day, and often end up with different side effects, which can range from nausea to vomiting, diarrhea, dizziness and headaches.

2.4.1 Omeprazole

Omeprazole is a proton pump inhibitor that suppresses gastric acid secretion by specific inhibition of the H+/K+- ATPase in the gastric parietal cell. By acting specifically on the proton pump, omeprazole blocks the final step in acid production, therefore decreasing gastric acidity. The structure of omeprazole is shown in Figure 2.3.

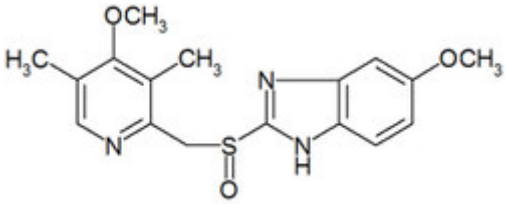

Figure 2.3: Chemical structure of omeprazole 5-methoxy-2-[(4-methoxy-3,5-dimethyl-pyridin-2-yl)methylsulfinyl]-3H-benzoimidazole (Singh, 2008).

2.4.2 Characteristics of Omeprazole

Omeprazole has some unique features and charcteristics that are important for the way it works. First of all, omeprazole is lipophilic and can easily penetrate cell membranes. Second, omeprazole is a weak base, which means that it concentrates in acid compartments. Third, omeprazole is unstable in an acidic solution. The half-life of omeprazole at pH 1 is approximate two minutes; yet when it is exposed to a neutral solution, that is pH 7.4; it can last for about 20 hours. Omeprazole is thus a drug that accumulates within the acid space of the target cell, where it is transformed into an active inhibitor.

2.4.3 Limitations of Omeprazole

Toxicological studies using very high doses of omeprazole in rats showed that this could lead to the formation of endocrine tumours (carcinoids) in the stomach. The carcinoids arose from entero-chromaffine-like (ECL) cells, a type of endocrine cell in the gastric mucosa that synthesizes and produces histamine, a process stimulated by the gastric hormone gastrin (Larsson *et al*., 1986). At the same time, longer term stimulation by gastrin has a massive effect on ECL cells. As the amount of gastrin increases and acid secretions decrease, this effect can result in the elimination of gastric acid secretions, leading to massive hypergastrinemia (Mattsson *et al*., 1991).

2.5 Mushrooms

2.5.1 Introduction to mushrooms

Nowadays, there are at least 80,060 species of described fungi of all kinds on the Earth. This figure is based on the total derived from adding together the species in each genus given in the latest edition of the Dictionary of Fungi (Kirk *et al*., 2001), which includes all organisms traditionally studied by mycologists: slime molds, chromistan fungi, chytridiaceous fungi, lichen-forming fungi, filamentous fungi, moulds and yeasts.

From the definition of Chang and Miles (1992), 'a mushroom is a macrofungus with a typical fruiting body which can be hypogeous or epigeous, large enough to be seen with the naked eye and to be picked by hand'. The number of filamentous fungi that are mushrooms, in the sense of this definition deduced from the Dictionary of the Fungi, is at least 14,000 and perhaps as many as 22,000 (Hawksworth, 2001). That, the actual number of such species on Earth is probably much higher is due to two main reasons: (1) the large number of as yet undescribed species; and (2) the fact that many morphologically defined mushroom 'species' prove to be assemblages of several biological species (Hawksworth, 2001). Potentially new mushrooms are regularly found in the tropics, especially species forming ectomycorrhizas with native trees. In various tropical areas, 22–55% (in some cases up to 73%) of mushroom species have turned out to be undescribed (Hawksworth, 2001). An analysis of the localities from which fungi new to science have been described and catalogued in the Index of Fungi in the 10 years from 1990 to 1999 revealed that about 60% of all newly identified fungi were from the tropics (Hawksworth 1993, 2001); and this is also the case for mushrooms, although new species also continue to be discovered in Europe and North America.

Researches of compatibility and molecular sequences between mushrooms previously considered to be the same species on morphological grounds have revealed

'cryptic species', i.e., populations functioning as separate biological species but covered by a single scientific name. Hawksworth (2001) stated that a single morphologically identified species may consist of 20 or more biological species. Given this, the number of fungi species on Earth could range from 500,000 to as many as 9.9 million, of which only 80,060 have been named. A working figure of 1.5 million species is generally accepted, and new data suggests that this is not unreasonable. Within this, the number of mushrooms on Earth is estimated at 140,000, of which maybe only 10% are known. Meanwhile, of those nearly 14,000 species that we know today, around 50% are considered to have varying degrees of edibility; just over 2,000 are safe; and about 700 are known to possess significant pharmacological properties (Chang, 1999; Wasser & Weis 1999; and Reshetnikov *et al*., 2001). Hence, it is clear that mushrooms represent a major potential source of powerful new pharmaceutical products.

2.5.2 *Hericium erinaceus*

Hericium erinaceus (Bull. Fr.) Pers. is known as monkeyhead mushroom in China, and 'cendawan bunga kobis' in Malaysia. Other common names for *Hericium* spp. included lion's mane, old man's beard, hedgehog mushroom, satyr's beard, pom pom and yamabushitake (Japanese for "mountain-priest mushroom")(Stamets, 2005).

The basidiocarp of *H. erinaceus* appears as a slightly rounded structure, up to 25 cm in diameter; pure white when fresh, but easily becoming bruised and staining yellowish brown (Figure 2.4). There is no recognizable cap, but only a short stalk from which grows a cascade of long, downwardly projecting spines, up to 4 cm long. In nature, the fruit body always grows out of the knotholes or wounds of a tree, which may be walnut, oak, elm, or beech. In the wild, only the fresh, young fruit body is edible and is regarded as a delicacy- an especially desirable one, as the fruit body can be large.

When this mushroom becomes old and yellowish brown, the taste can turn bitter and unpleasant (Pegler, 2003).

Figure 2.4: Basidiocarp of *Hericium erinaceus.* (Source: retrieved from Prof. Dr. Vikineswary Sabaratnam, University of Malaya)

Because of the rapid increase of interest in *Hericium* species, more information about this mushroom is necessary to screen efficient species and strains, and to improve its yield and quality. Unfortunately, limited data and reference texts are available on the physiological, genetic, and cultural characteristics of the *Hericium* species. To date, only a few *Hericium* species have been identified throughout the world. Park *et al.* (2004) analyzed the phylogenetic relationships of seven *Hericium* species including *H. abietis, H. alpestre, H. americanum, H. coralloides, H. erinaceum, H. erinaceus* and *H. laciniatum*. Among the seven *Hericium* species, attempts at cultivation on logs, stumps and sawdust have been made so far only with *H. erinaceum* (Suzuki & Mizuno, 1997), *H. erinaceus* (Chang & Roh, 1999) and *H. abietis* (Xiao & Chapman, 1997). Ko *et al.* (2005) studied the potential for using agricultural by-products as a supplementary resource of sawdust substrate for the growth of seven *Hericium* species, as well as basidiomata formation and production on oak sawdust substrate.

2.5.3 Medicinal properties of *H. erinaceus*

Mizuno (1999) found that polysaccharides (HEPS) in the fruiting bodies of *Hericium erinaceus* may have beneficial effects against stomach, esophageal and skin cancers. *Hericium erinaceus* (including its fruiting body, mycelium, and products in the medium) also contains some lower-level MW pharmaceutical constituents, such as the novel phenols (hericenones A and B) and Y-A-2, which may have chemotherapeutic effects on cancer. Yang *et al.* (2003) studied the hypolipidaemic effect of an exo-biopolymer produced from a submerged mycelial culture of *H. erinaceus* in dietary-induced hyperlipidaemic rats. After oral administration of exo-biopolymer at a dose of 200 mg/kg of body weight, the plasma total cholesterol (32.9%), low density lipoprotein cholesterol (45.4%), triglyceride (34.3%), phospholipids (18.9%), atherogenic index (58.7%) and hepatic HMG-CoA reductase activity (20.2%) were extensively reduced. It also increased the plasma high density lipoprotein cholesterol level (31.1%) over that of the control group (Yang *et al*., 2003).

Recent studies have determined that *H. erinaceus* may have important physiological functions in humans, including antioxidant activities, the regulation of blood lipid levels and reducing blood glucose levels (Chyi *et al*., 2005). Gue *et al*. (2006) stated that *H. erinaceum* is a medicinal and edible mushroom with anti-microbial and anti-cancer activities.

Recently, *Hericium* spp. has attracted considerable attention from researchers owing to its antimicrobial effects (Kim *et al*., 2000), anti-tumor activities (Mizuno *et al*., 1995), immunomodulatory effects (Liu *et al*., 2002), antioxidant properties (Mau *et al*., 2002), cytotoxic effects (Kuwahara *et al*., 1992), and promotion of synthesis of neurogrowth factors (Kawagishi *et al*., 1994).

2.5.4 Nutritional composition of *H. erinaceus*

Wang *et al*. (1992) studied the nutritional composition of five strains of *H. erinaceus* fruit bodies that have the highest crude protein content (28.4%) and low crude fat. Kim *et al.* (2011) found that, for four *H. erinaceus* extracts, protein, carbohydrate, and mineral (ash) content were all high, ranging (in % of dry weight) from 35.5 to 38.5; 33.8 to 39.5 and 14.6 to 19.0 respectively; moisture content was about 8%; while fiber and fat content were <1%.

2.6 Free radicals

2.6.1 Introduction to free radicals

A free radical is any chemical species capable of independent existence that has one or more unpaired electrons. Free radicals are often described by the insertion of a radical dot (·) to indicate that one or more unpaired electrons is present. Usually, radicals are less stable than nonradicals, in that they can interreact either with another radical or with another molecule in various ways to achieve a stable state.

2.6.2 Reactive oxygen species

Aerobes require oxygen (O_2) to perform cell functions, as oxygen plays an important role in a series of biochemical reactions such as electron transport chains and other enzyme systems. A series of electron transfer reactions produces several molecules of adenosine triphosphate (ATP), which in turn provides the energy required for a multitude of cellular reactions and functions for each electron that passes through the respiratory chain. Molecular oxygen can pair with four electrons, one at a time, and

the corresponding number of protons to yield two molecules of water. Different oxygen radicals are formed as intermediate products during this process, namely superoxide (O_2^-); peroxide ($O_2^{=}$), which normally exists in cells as hydrogen peroxide (H_2O_2); and the hydroxyl radical (•OH). Superoxide, peroxide, and the hydroxyl radical are considered the primary reactive oxygen species (ROS). Reactive oxygen species (ROS) is often used to refer not only to the radicals $OH\cdot$, $RO_2\cdot$, $NO\cdot$ and $O_2\cdot^-$, but also the nonradicals HOCl, $^1O_2$, $ONOO^-$, O_3, and H_2O_2.

An imbalance between the production of ROS and the activity of the antioxidant defences is called oxidative stress. Oxidative stress, superoxide production and an imbalance in antioxidant enzymes may contribute to the initiation of cancer (Wiseman & Halliwell, 1996), cause structural alterations in DNA (Halliwell & Aruoma, 1991), affect cytoplasmic and nuclear signal transduction pathways (Burdon, 1995).

2.6.3 Antioxidants

An antioxidant is defined as any substance that, when present at low concentrations compared with those of the oxidizable substrate, considerably delays or inhibits oxidation of the substrate (Gutteridge, 1995). Antioxidants can act by: (a) removing oxygen or decreasing local oxygen concentration; (b) removing catalytic metal ions; (c) removing key reactive oxygen species such as superoxide and hydrogen peroxide; (d) scavenging initiating free radicals such as hydroxyl, alkoxyl, and peroxyl species; (e) breaking the chain of an initiated sequence; or (f) quenching or scavenging singlet oxygen (Gutteridge, 1995).

2.6.4 Antioxidant compounds

Vitamin C (ascorbic acid) is a major aqueous-phase antioxidant, and Vitamin E (α-tocopherol) is a major membrane bound antioxidant. These compounds are both human vitamins that play vital roles in maintaining health.

There are two types of antioxidant: water-soluble and lipid-soluble antioxidant compounds. Water-solubles include uric acid, glutathione and ceruloplasmin. Uric acid is the end product of purine metabolism in humans, and may function both as a classic suicidal antioxidant and as a chelator of transition metals. Uric acid may inhibit metal-catalyzed oxidation reactions without itself becoming oxidized, by binding iron and/or copper. Beta-carotene and ubiquinone are lipid-soluble agents that may also play important antioxidant roles *in vivo*.

2.6.5 Antioxidant Enzymes

In order to minimize oxidative damage, aerobic organisms also produce numerous antioxidant enzymes (Fridovich, 1995; and Özben, 1998). One of the best known of these enzymes is superoxide dismutase (SOD).

Superoxide dismutase (SOD) catalyzes the dismutation reaction of $O_2^- + O_2^-$ $\longrightarrow$ $H_2O_2+O_2$ and increases the rate constant of this important dismutation reaction several-fold. SOD is found in almost all eukaryotic cells, including yeasts, plants and animals. Different members of the SOD family use different transition metals such as Ferum, Manganese, Copper/ Zinc at their active sites. Bacteria use a Fe-SOD and a Mn-SOD, whereas mammals apply distinct cytoplasmic and extracellular forms of Cu/Zn-SOD and a mitochondrial Mn-SOD which is closely related to bacterial Mn-SOD. Mn-SOD has four subunits, with Mn in each subunit (Smith & Heath, 1976). Cu/Zn-SOD consists of two subunits, with each unit having an active site containing one Cu ion and

one Zn ion (McCord, 1979). Cu ion serves as an active redox site while Zn maintains the protein structure (Fridovich, 1975). Cu/Zn-SOD is found mainly in cytosol and is shown to be present in the nucleus also (Slot *et al*., 1986). SOD present in our body can dismutate O_2^- rapidly at the site where it is formed.

The product of SOD is H_2O_2, which is clearly toxic and must be quickly removed. In mammalian cells this is accomplished by two enzyme families: the glutathione peroxidases and the catalases. Both glutathione peroxidases and catalases detoxify H_2O_2 by reducing it to water and oxygen. Glutathione peroxidase is not further discussed here.

Catalase removes H_2O_2 and generates O_2, $2H_2O_2 \longrightarrow 2H_2O + O_2$ (Krall *et al*., 1988). In most mammalian cell types, catalase is only found within peroxisomes where it has a clear function of removing the H_2O_2 generated by β-oxidation of long-chain fatty acids. Normally, catalase is not found in the cytoplasm of most mammalian cells, and for that reason diffusion of H_2O_2 from the cytoplasm into peroxisomes seems rather unconvincing all of which suggests that glutathione peroxidases largely deal with cytoplasmic H_2O_2 and catalases largely deal with peroxisomal H_2O_2.

2.6.6 Lipid peroxidation activity

The free-radical oxidation of polyunsaturated fatty acids in biological systems is known as lipid peroxidation. Lipid peroxidation is an inevitable process *in vivo.* Cardiovascular diseases such as preeclampsia and atherosclerosis are induced by this process, and the end-products of this process such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE) can cause damage to proteins and to DNA. Lipid peroxidation causes damage to biological membrane functions, e.g. it decreases fluidity, inactivates

membrane-bound enzymes and receptors, and may alter nonspecific calcium ion permeability (Orrenius, 1989; and Bast, 1993).

Lipid peroxidation induced by oxygen free radicals is believed to play an important part in the destruction and impairment of cell membranes, because this process degrades polyunsaturated fatty acids (PUFA) in the latter, with consequent disruption of membrane integrity (Tappel, 1973).

Previous studies have reported that lipid peroxidation plays a significant role in the pathogenesis of gastric mucosal lesions induced by water immersion restraint stress, burn shock, and ischemia-reperfusion (Yoshikawa *et al*., 1986, 1987, 1989).

2.7 Heat shock proteins

Heat shock response was first reported by Ritossa (1962), who observed it in *Drosophila melanogaster* chromosomes after subjecting these flies to heat treatment. A heat shock response then increases the expression of genes encoding a special class of proteins, called heat shock proteins (HSPs) or stress proteins.

Heat shock proteins play important roles in both normal conditions and pathological situations involving either systemic or cellular stress. Previous studies have shown that most HSPs have strong cytoprotective effects, are involved in many regulatory pathways, and act as molecular chaperones to preserve important cellular proteins (Hightower, 1991, and Arya, 2007). HSPs are essential for the maintenance of gastric mucosal cell integrity both during normal cell growth and in several pathophysiological conditions (Robert *et al*., 1983; Lichtenberger, 1999; and Laine *et al*., 2008).

Heat shock proteins are also implicated in gastric cytoprotection. HSPs are a group of highly conserved proteins that are induced in response to heat and other

stresses (Kinouchi *et al*., 1994; and Schiaffonati & Tiberio, 1997). They are classified into subfamilies according to their molecular weight, such as small HSPs (16–30 kDa), HSP40, the predominantly mitochondrial HSP, HSP60, HSP70, HSP90 and the large HSP110. 70 kDa heat shock proteins assist in both the assembly and transport of newly synthesized proteins within cells and also the removal of denatured proteins. Therefore, HSP70 plays a role in preventing damage and in cellular repair processes after injury. Previous studies have also found the induction of HSP70 in response to heat, ethanol, oxidative stress, or water immersion–restraint stress in the gastric mucosal cell (Zeniya *et al*., 1995; and Hirakawa *et al*., 1996). However, only a few studies have reported locating HSP70 in the injured stomach.

2.7.1 Heat shock proteins and the stomach

The stomach is one of the organs in our body that is often exposed to outside irritants such as hot food, alcohol, and oxidants generated from ingested food and *H. pylori*-associated infection. Alcohol is one of major factors that cause gastric mucosal injuries including gastritis or ulcers.

Some studies showed that HSPs contribute to adaptive cytoprotection in the gastrointestinal mucosa. Among the HSPs, HSP70 is believed to be the one that plays an important role in the defense mechanism of gastrointestinal mucosa. HSPs are reported to have been induced both in *in vitro* and *in vivo*. According to Nakamura *et al*. (1991), HSPs were induced by heat shock treatment resistant 7.5% ethanol in cultured guinea pig gastric mucosal cells. In an *in vivo* study, it was reported that an overexpression of HSPs was induced by water-immersion restraint (WIR) stress in animals pretreated with hyperthermia, as compared with stress alone (Itoh & Noguchi, 2000). Hirakawa *et al*. (1996) revealed that the oral administration of the anti-ulcer drug

geranylgeranylacetone (GGA) produced HSP60, HSP70, HSC70, and HSP90 in the gastric mucosa of normal rats. Park *et al*. (2008) also demonstrated that HSP70 inductions protected rats against ethanol-induced gastric mucosal damages.

These results suggest that HSPs, especially HSP70, induce resistance in the gastric mucosa against stress-induced mucosal damage. HSPs thus play a protective role in gastric mucosa under stressful conditions.

2.7.2 Heat shock proteins and protection against oxidative stress

Heat shock proteins also play an important role in other cellular processes that occur during and after exposure to oxidative stress. Oxidative stress occurs as a result of an imbalance between the productions of reactive oxygen species (ROS) and the detoxifying process of reactive intermediates. As a consequence, this imbalance generates excessive ROS, which leads to the oxidation and aggregation of vital proteins and of DNA, causing a failure of normal cell functions.

Under such stress conditions, some HSPs, especially the HSP70 family and its co-chaperones, are synthesized at high levels. Members of the HSP70 protein family include: HSC70 (a constitutive HSP70), present within the cytoplasm and nucleus; grp75, a mitochondrial HSP70; and grp78 (Bip), a resident of the endoplasmic reticulum.

The HSP70 protein family functions as molecular chaperones in refolding denatured polypeptide (Gething & Sambrook, 1992; Hendrick & Hartl, 1993; Becker & Craig, 1994; and Hartl, 1996). A molecular chaperone is defined as a protein that binds to newly synthesized proteins and refolds denatured proteins (Bukau & Horwich, 1998; and Young *et al*., 2004).

Members of the HSP70 family and its co-chaperones select and direct abnormal proteins to the proteasome or lysosomes for degradation (Mayer & Bukau, 2005). Previous studies have revealed that an overproduction of HSP70 can reduce stress-induced denaturation as well as the aggregation of certain proteins (Kampinga *et al*., 1995; and Kabakov & Gabai, 1995) - further evidence that HSP70 plays an important role in protection against stresses (Georgopoulos & Welch, 1993; and Kampinga, 1993). Heat shock proteins may also exhibit its protective effects against oxygen radical-induced cellular damage such as membranes (lipid peroxidation), proteins, DNA, and mitochondria. Jacquier-Sarlin *et al*. (1994) reported the protective effects of HSP70 against lipid peroxidation and DNA damage. The over-expression of HSPs may therefore protect multiple cellular compartments and prevent protein damage from oxidative stress.

2.8 Bax protein

The balance between apoptosis and cellular proliferation is a main factor in gastric injury and repair. These processes are regulated by several genes, including p53 and members of the Bcl-2 family such as Bax and Bcl-2 (Haunstetter & Izumo, 1998; Boucher *et al*., 2000; and Rezvani *et al*., 2000).

The Bax gene is a proliferative suppressor gene that encodes the Bax protein which promotes apoptosis. Bax is a protein that forms an isomeric dimmer with Bcl-2. It counteracts the action of Bcl-2 in the presence of various cell death signals, and induces apoptosis (Wu *et al.*, 1998*).* Bax protein expression has been identified in various human tissues such as the prostate, colon, breast, testis and ovary. However, little is known about Bax protein expression in gastric injury. Qiao *et al*. (2011) reported high levels of expression of Bax protein in the early stages of the gastric

ischemia reperfusion of rats, which decreased gradually after that. This suggests that a down-regulation of Bax protein is related to healing effects in gastric ulcers.

3.0 Materials and methods

3.1 Mushroom and preparation of mushroom extracts

One batch of 20kg fresh basidiocarp of *H. erinaceus* KUM 61131 was collected from Highland & Lowland Mushroom Industry, Selangor, Malaysia. Fresh *H. erinaceus* fruit bodies were sliced and frozen at -20 ± 2 °C prior to freeze-drying at -50 ± 2 °C.

The extraction was based on the method by Wong *et al*. (2007). Freeze-dried fruit bodies of *H. erinaceus* were blended into powder. The yield was 13.57%. The powdered fruit bodies were soaked with distilled water at a ratio 1:10 (w/v) and left for 24 h at 27 ± 2°C. The soaked fruit bodies were then boiled for 30 min with agitation, left covered for 30 minutes, and filtered cold using Whatman no.1 filter paper. The extract was then freeze-dried. The yield was 27.47%.

3.2 The toxic effects, if any, of extracts freeze-dried fruit bodies of *H. erinaceus* to rats

3.2.1 Animals

Healthy male and female *Sprague Dawley* rats weighing between 150 g and 300 g were used in this study. The female animals used were nulliparous and non-pregnant. The animals were fed with pellet feed. Food and water were provided ad libitum during acclimation and throughout the study. The animals were maintained under control condition of temperature at 26°C (±2°C) and relative humidity around 44–56%. Animals were housed in polypropylene cages over husk beddings and 12 h light and 12 h dark cycle was maintained throughout the experimental period. All the animal experiments were performed after getting necessary approval from the Institutional

Animal Ethical Committee (which follows the guidelines of Animal Care and Use Committee), Laboratory Animal Science Centre, Faculty of Medicine, University of Malaya (Ethics no. ISB/11/08/2010/WJY (R). All efforts were made to minimize both the number of animals used and unwanted stress or discomfort to the animals throughout the experimental procedures.

3.2.2 Acute toxicity studies

The acute toxic study was used to determine a safe dose of the mushroom extract. Animals were divided into three groups, each group containing six animals. Group 1 animals served as control which received distilled water. Group 2 and Group 3 animals received freeze-dried *H. erinaceus* powder in single doses of 2 and 5g/kg b/w., respectively. The *H. erinaceus* powder was suspended in sterile distilled water and administered by gavage (P.O.). Food was withheld for a further three to four hours after treatment (OECD, 2001). The animals were observed for 30 min and 2, 4, 24 and 48 h for the onset of clinical or toxicological symptoms. Mortality, if any was observed over a period of two weeks. During this period, body weight was recorded weekly to evaluate possible toxic effect. After an overnight fast, the animals were sacrificed on the 15^{th} day. Hematological, serum biochemical and histological (liver and kidney) parameters were determined using standard methods. The experimental flow is given in Figure 3.1.

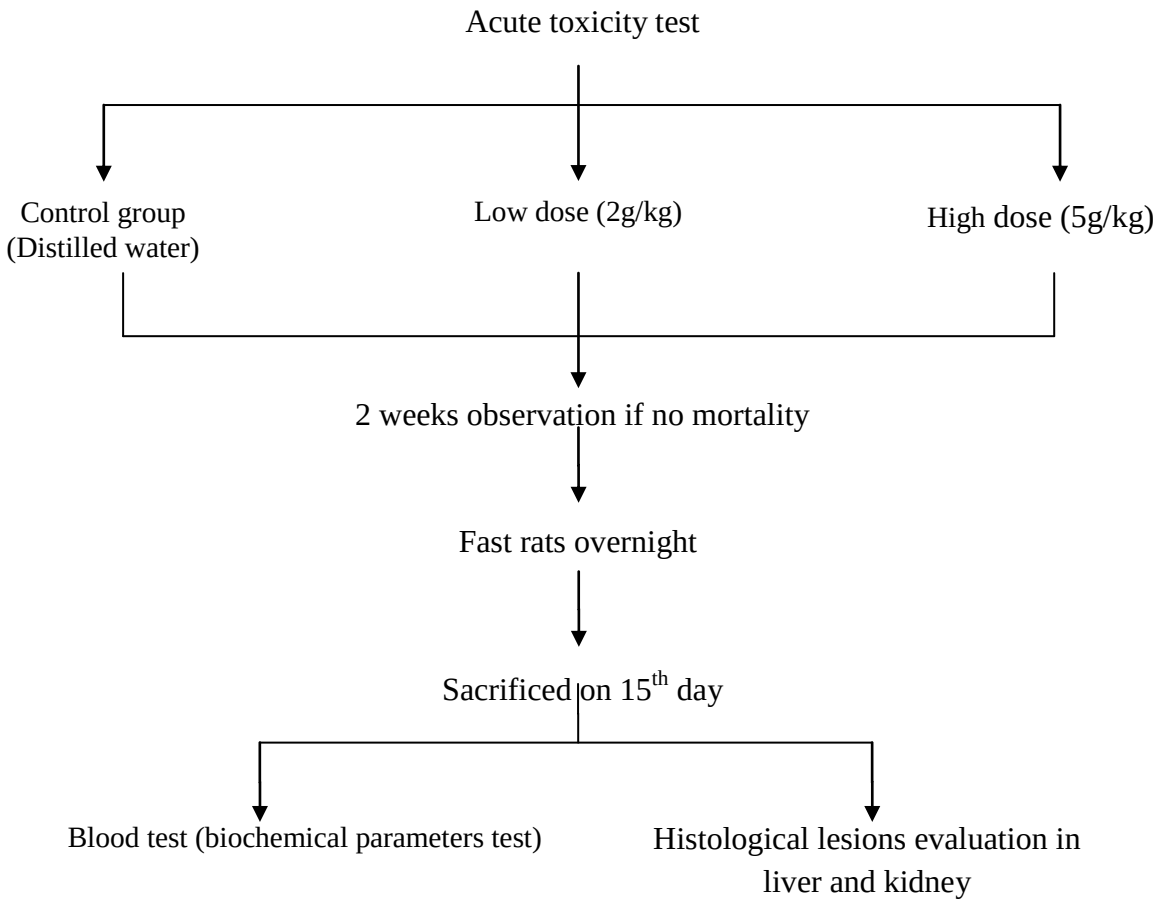

Figure 3.1: Flow diagram of the acute toxicity studies.

3.3 The gastroprotective effects of *H. erinaceus* extract in rats

The rats were starved for 24 hours (Garg *et al*., 1993) but had free access to drinking water until two hours before the experiment. On the day of experiment, the rats were randomly divided into seven groups with six animals. Each group received oral dose of distilled water (ulcer-free control group), distilled water (ulcer control group), four different concentrations of mushroom extracts (50, 100, 200, and 400mg/kg b/w., respectively) in distilled water and Omeprazole in distilled water (positive control group) were the treatments to investigate gastroprotective activities, if any, of aqueous extracts of *H. erinaceus* (Figure 3.2). All groups were orally fed with 95% alcohol (De Pasquale *et al*., 1995) one hour after their pre-treatment, except ulcer-free control group which

was given distilled water, 5ml/kg. The animals were under mild ether anaesthesia. The pyloric portion of stomach was identified, slightly lifted out and ligated, avoiding traction to the pylorus or damage to the blood supply. The stomach was then replaced carefully and abdominal wall was closed by interrupted sutures. Four hours after ligation, all animals were sacrificed. The stomach was cut along the greater curvature and separated from the surrounding tissues and thus brought out as a whole along with its contents. The content (gastric juice) was subjected to centrifugation at 3000 rpm for 10 min and the pellate was then analysed for mucous content. Tissues were homogenized with phosphate buffer saline (PBS). Both serum and tissue homogenates were stored at -80°C until further analysis.

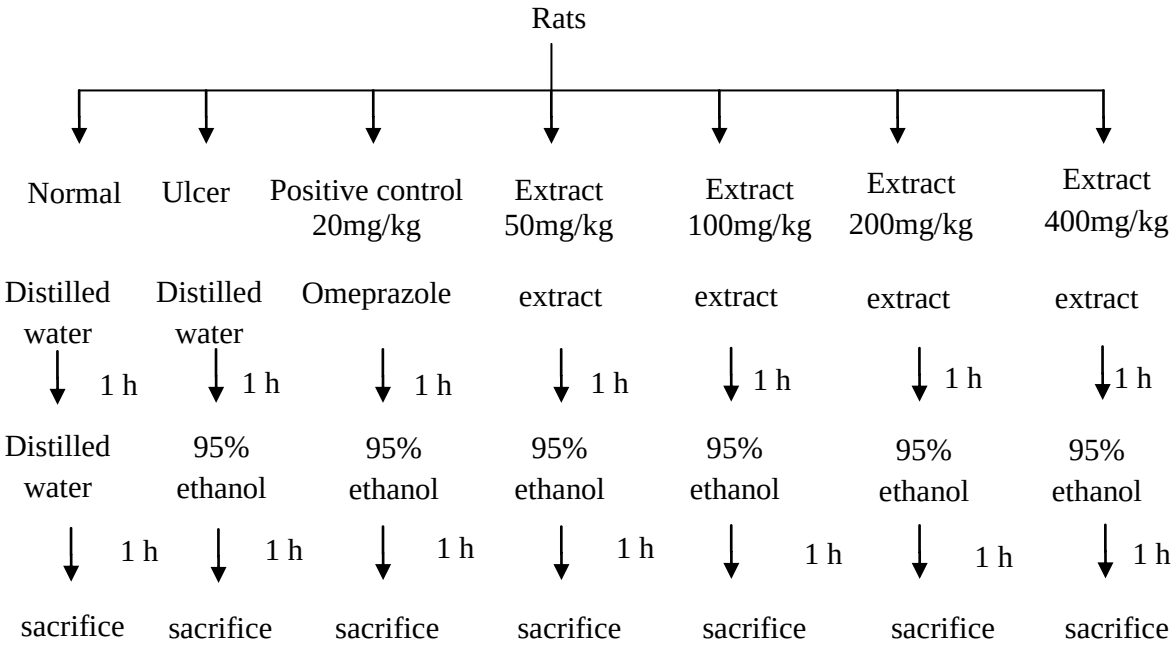

Figure 3.2: Flow diagram of the gastricprotective studies (Abdulla *et al.,* 2008).

3.4 The ulcer healing effects of *H. erinaceus* extract in rats

The ulcer healing activity of *H. erinaceus* was performed in ethanol induced ulcer model (Figure 3.3). In this test, the two concentrations of the mushroom extracts that showed best results were used in ulcer treatment tests. The rats were starved for 24 hours (Mohamad Omar *et al*., 2011) but had free access to water until two hours before the experimentation. The rats were randomly divided into five groups: control group (no treatment), positive group (treated with Omeprazole), negative group (treated with distilled water), low dose group (200mg/kg) and high dose group (400mg/kg). Each groups contained six animals. On the first experiment day, the control group was sacrificed without any treatment. The remaining groups were provided with food and water prior to the experiment day and this was followed by the respective treatments for three days. All animals were anaesthetised with ketamine and sacrificed; stomachs were collected for histological analysis (Wasman *et al.*, 2010).

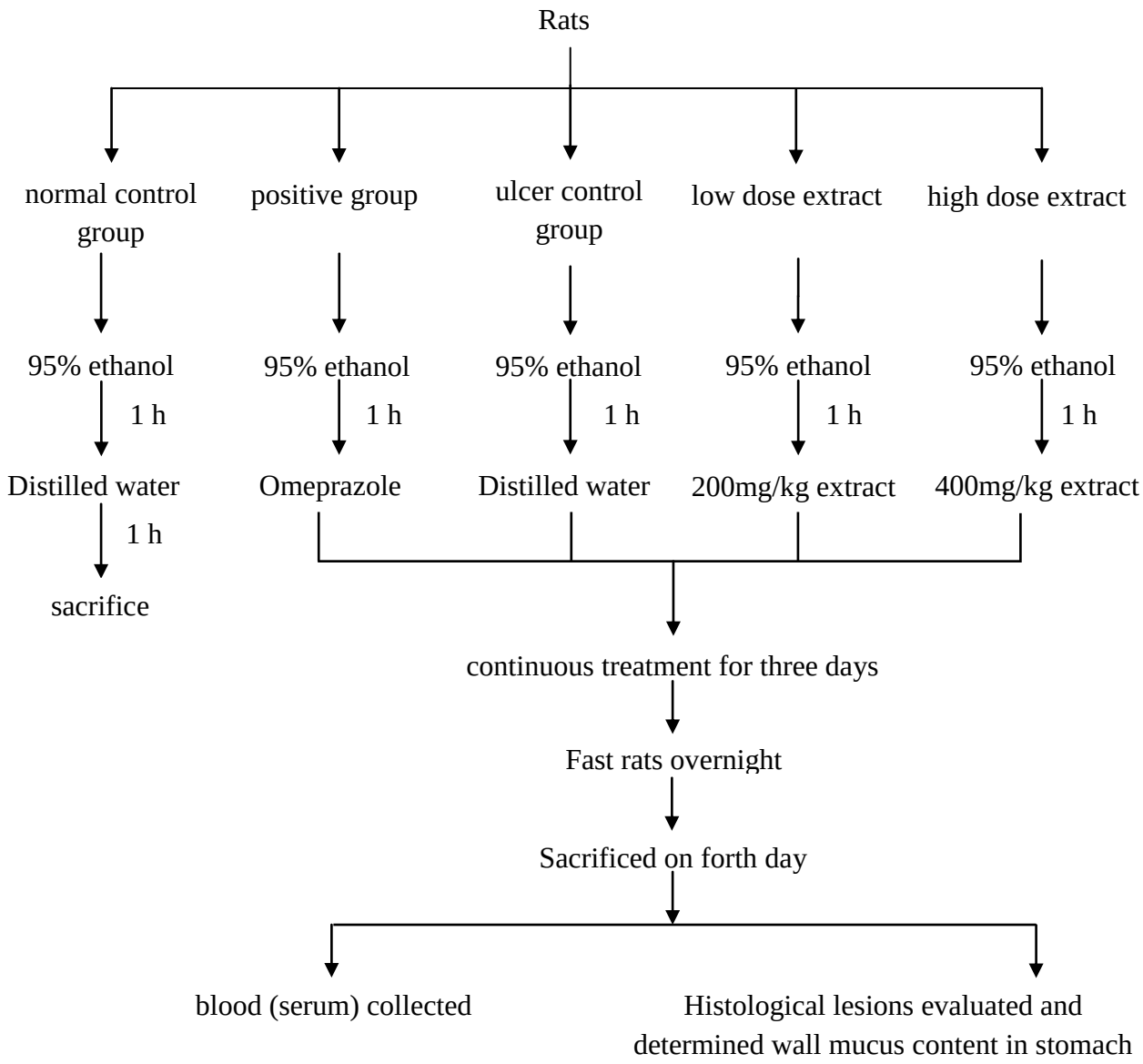

Figure 3.3: Flow chart for ulcer treatment tests (Mohamad Omar *et al*., 2011).

3.5 Gross gastric lesions evaluation

Ulcers were found in the gastric mucosa, appearing as elongated bands of hemorrhagic lesions parallel to the long axis of the stomach. Each gastric mucosa was examined for damage. The length (mm) and width (mm) of ulcer on gastric mucosa

were measured by a planimeter (10 × 10 mm^2 = ulcer area) under dissecting microscope (x1.8).

The area of each ulcer lesion was measured by counting the number of small squares, 2 × 2 mm, covering the length and width of each ulcer band. The sum of the areas of all lesions for each stomach was applied in the calculation of the ulcer area (UA) wherein the sum of small squares × 4 × 1.8 = UA mm^2 as described by Kauffman and Grossman (1978) with slight modification. The inhibition percentage (I %) was calculated by the following formula as described by Njar *et al*. (1995) with slight modification.

(I %) = [(UAcontrol − UAtreated) ÷ UAcontrol] × 100%.

3.6 Measurement of mucus production

Gastric mucus production in the rats that were subjected to ethanol-induced gastric mucosal injury was measured. The gastric mucosa of each rat was gently scraped using a glass slide and the mucus obtained was weighed using a precision electronic balance (Tan *et al*., 2002).

3.7 Gastric wall mucus determination

The modified method of Corne *et al*. (1974) was used to determine gastric wall mucus. The glandular segments from stomach, which had been opened along their greater curvature, were weighed. All weighed segments were transferred immediately to 10 ml of 0.1%, w/v alcian blue solution (in 0.16 M sucrose solution, buffered with 0.05 M sodium acetate adjusted to pH 5.8 with HCl). Segments were stained for two hours, excess dye was removed by two successive rinses with 10 ml of 0.25 M sucrose, first for 15 min and then for 45 min. Alcian blue complexed with the gastric wall mucus was extracted with 10 ml of 0.5 M magnesium chloride, by shaking for one min at 30 min

intervals for two hours. The resulting blue solution was shaken vigorously with an equal volume of diethyl ether and then the emulsion was centrifuged at 3000 rpm for 10 min and the absorbance of the aqueous layer against blank standard $MgCl_2$ solution was recorded at 580 nm. The quantity of alcian blue recovered from per gram of net glandular tissue was then calculated.

3.8 Histological evaluation of gastric lesions

Specimens of the gastric walls from each rat were fixed in 10% buffered formalin and processed in a paraffin tissue processing machine. Sections of the stomach were made at a thickness of 5μm and stained with hematoxylin and eosin for histological evaluation.

Histological analyses were made using a Nikon microscope with NIS-Elements D Imaging Software 2.30 version. The images were manipulated in ACDSee Version 10.0 Photo Manager to enhance the image resolution and to frame the plates.

3.9 Immunohistochemistry

Tissue section slides were heated at 60°C for approximately 25 min in hot air oven (Venticell, MMM, Einrichtungen, Germany). The tissue sections were deparaffinized in xylene and rehydrated using graded alcohol. Antigen retrieval process was done in 10mM sodium citrate buffer boiled in microwave. Immunohistochemical staining was performed according to manufacturer's protocol (Dakocytomation, USA). At first, endogenous peroxidase was blocked by peroxidase block (0.03% hydrogen peroxide containing sodium azide) for five min. Tissue sections were washed gently with wash buffer, and then incubated with HSP70 (1 : 500) and Bax (1 : 200) biotinylated primary antibodies for 15 min. The sections were rinsed gently with wash buffer and placed in buffer bath. The slides were then placed in a humidified chamber

and sufficient amount of streptavidin-HRP (streptavidin conjugated to horseradish peroxidase in PBS containing an antimicrobial agent) was added and incubated for 15 min. After that, tissue sections were rinsed gently in wash buffer and placed in buffer bath. Diaminobenzidined (DAB-) substratechromagen was added to the tissue sections and incubated further for five min following washing and counterstaining with hematoxylin for five second. The sections were then dipped in weak ammonia (0.037 mol/L) 10 times, then rinsed with distilled water and covered with cover slip. Positive findings of the immunohistochemical staining should be seen as brown stains under light microscope.

3.10 Estimation of free radical generation

3.10.1 Lipid peroxidase (LPO)

The LPO assay was assessed based on a method described by Maelinda *et al*. (2008) but with minor modifications. 0.25 ml of tissue homogenated was mixed with same amount of freshly prepared 15% TCA. The mixture was centrifuged at 3500 rpm for five minutes. 0.4 ml of supernatant was taken out and mixed with 0.5 ml of 1% TBA. The reaction tubes were kept in a boiling water bath for 30 minutes. Upon cooling, the tubes were centrifuged at 3500 rpm for five minutes to remove precipitated protein. The formation of TBARS was measured by removing 200 µl of supernatant and measuring the absorbance at 532 nm. Each assay was carried out in triplicate. The results are expressed as µM 1,1,3,3-tetraetoxypropane equivalent.

3.10.2 Superoxide dismutase (SOD) activity

Superoxide dismutase (SOD) was determined by using Superoxide Dismutase Assay Kit (Catalog no. 706002, from Cayman Chemical Company, Ann Arbor, MI 48108). Figure 3.4 shows the process:

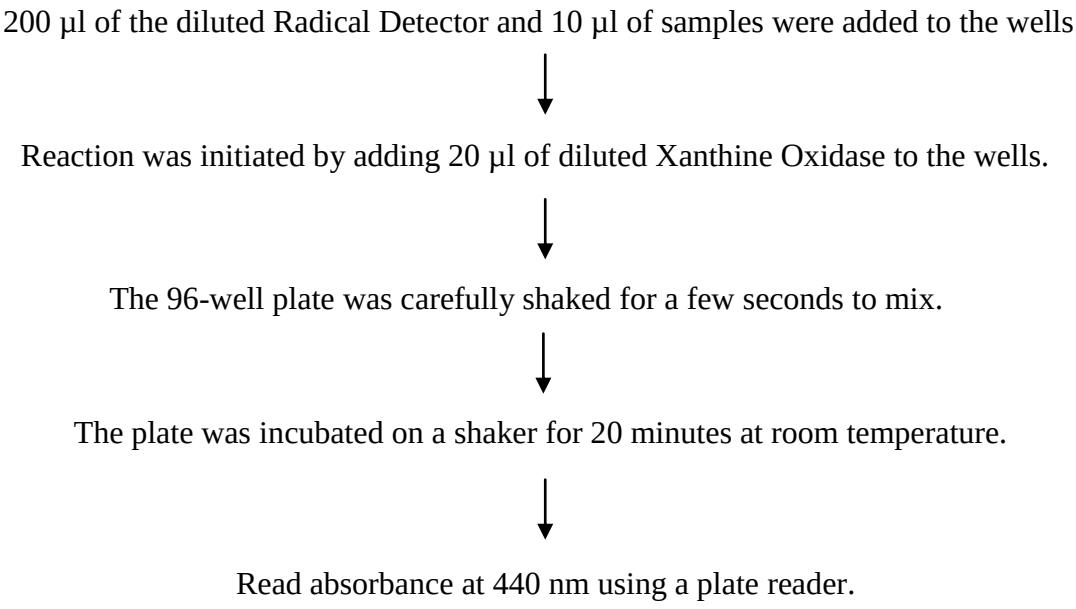

Figure 3.4: Superoxide dismutase (SOD) assay (SOD assay kit, Cayman Chemical Company).

3.10.3 Catalase (CAT) activity

Catalase (CAT) was investigated by using Catalase Assay Kit (Catalog no. 707002, from Cayman Chemical Company, Ann Arbor, MI 48108). The method was based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H_2O_2. The formaldehyde produced was measured colorimetrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) as the chromogen by using plate reader at a wavelength of 540nm. The process involved in the assay is shown below (Figure 3.5):

100 µl of diluted Assay Buffer, 30 µl of methanol, and 20 µl of sample were added to wells.

↓

20 µl of diluted Hydrogen Peroxide was added to all the wells to initiate the reactions.

↓

The plate was covered with plate cover and incubated on a shaker for 20 minutes at room temperature.

↓

30 µl of diluted Potassium Hydroxide was added to each well to terminate the reaction and then 30 µl of Catalase Purpald (Chromagen) was added to each well.

↓

The plate was covered with plate cover and incubated for 10 minutes at room temperature on a shaker.

↓

10 µl of Catalase Potassium Periodate was added to each well. The plate was covered with plate cover and incubated for five minutes at room temperature on a shaker.

↓

Read absorbance at 540 nm using a plate reader.

Figure 3.5: Catalase Assay (Catalase assay kit, Cayman Chemical Company).

3.11 Statistical analysis

All values were reported as mean ± S.E.M. Statistical analysis done using SPSS version 17.0. The significance of differences amongst groups was assessed using one-way ANOVA and Tukey multiple range test. A value of $p < 0.05$ was considered as statistically significant.

4.0 Results and Discussion

4.1 Acute oral toxicity studies on aqueous extracts of *H. erinaceus*

Animals treated with *H. erinaceus* extract at a doses of 2 and 5 g/kg were kept under observation for 14 days. Both sexes of rats showed no signs of toxicological effects during the experimental period after a single oral dose administration of the extract at 2 or 5 g/kg when compared to control group. Further, there was no mortality for rats administered with the extracts. In current study, all animals did not show any abnormal behavioural expressions and toxicological signs during experimental period.

4.1.1 Body weight of experimental rats

Evaluation of toxicity was carried out by observing body weight changes of the animals as shown in Figure 4.1 and Figure 4.2. During the two weeks period, body weight of male and female rats increased gradually from 0 day to 14 days in the three groups. Further, body weight of rats that received low or high dosage of *H. erinaceus* extract did not reveal significant differences when compared to animals in control group. The increases in body weight in male and female rats indicated that nutritional state of the animal was not compromised by the extracts. The results showed similarity to that obtained by Watthanachaiyingcharoen *et al*. (2009). However, it was not similar to the findings by Adebajo *et al*. (2006). Watthanachaiyingcharoen *et al*. (2009) showed that the body weight of both sexes' rats that received medicinal plant powder showed increase during the 14 days of observation period. However, Adebajo *et al*. (2006) found that rats that fed with *Murraya koenigii* leaf methanol extract at doses (250-450mg/kg) showed reduction in body weight and hepatic injury was observed after two

weeks experiment. This reduction in the body weight may be an indicator to evaluate the toxicity of selected natural compounds or extracts.

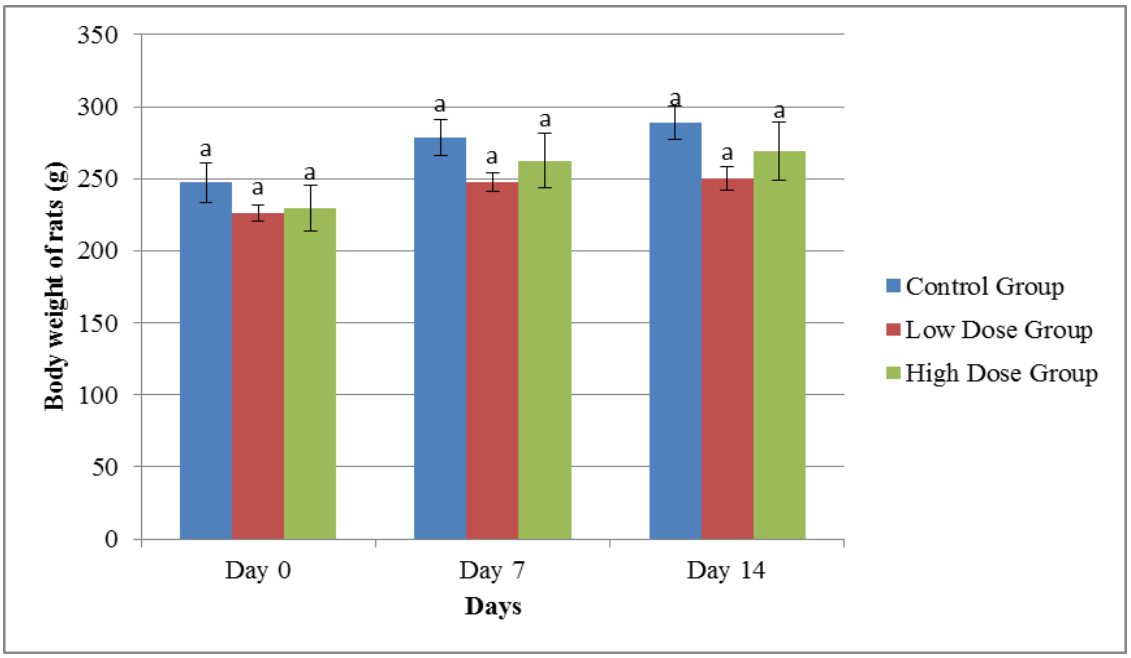

Figure 4.1: The effects of the aqueous extract of *H. erinaceus* on weight in the control and treated male rats during 14 days in the acute toxicity study. Means with same letters were not significantly different ($p < 0.05$) by Tukey Test. Six replicates animals were used for each treatment.

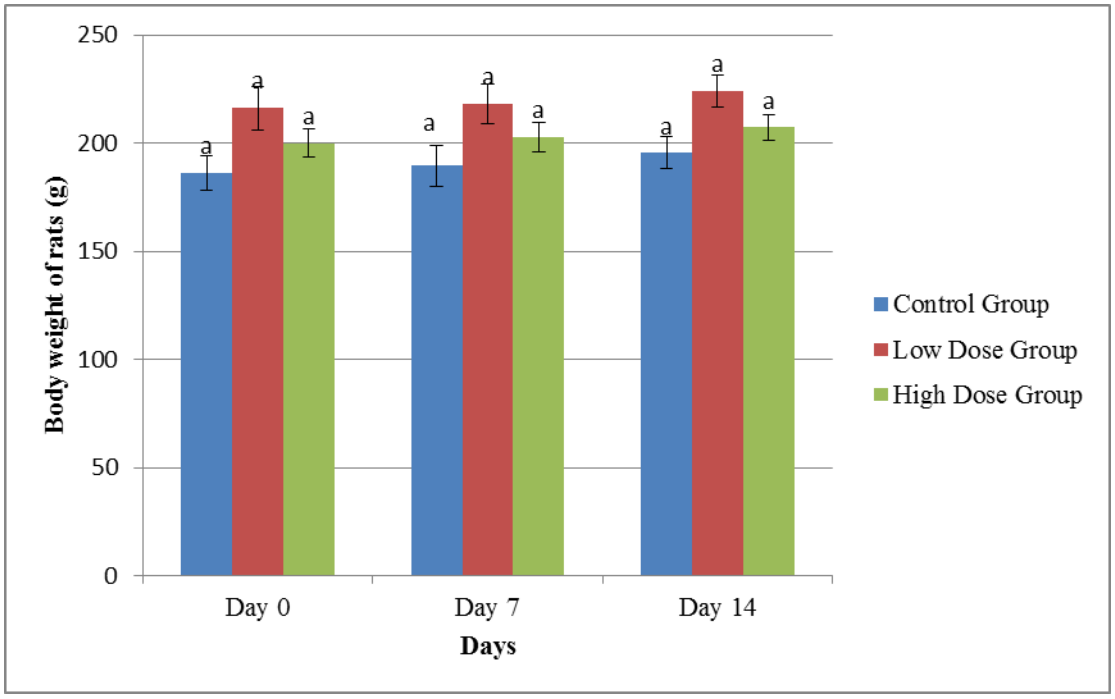

Figure 4.2: The effects of the aqueous extract of *H. erinaceus* on weight in the control and treated female rats during 14 days in the acute toxicity study. Means with same letters were not significantly different ($p < 0.05$) by Tukey Test. Six replicates animals were used for each treatment.

4.1.2 Histopathological examination of livers and kidneys of rats

No signs of morphological injury or lesion were found in the liver of rats treated with low and high doses of extracts. Normal cellular architecture with binucleated formation around the central veins was observed and the hepatocytes were arranged in cords (Plate 4.1a-c). These results showed that the *H. erinaceus* extract did not alter the physiological activities of the liver.

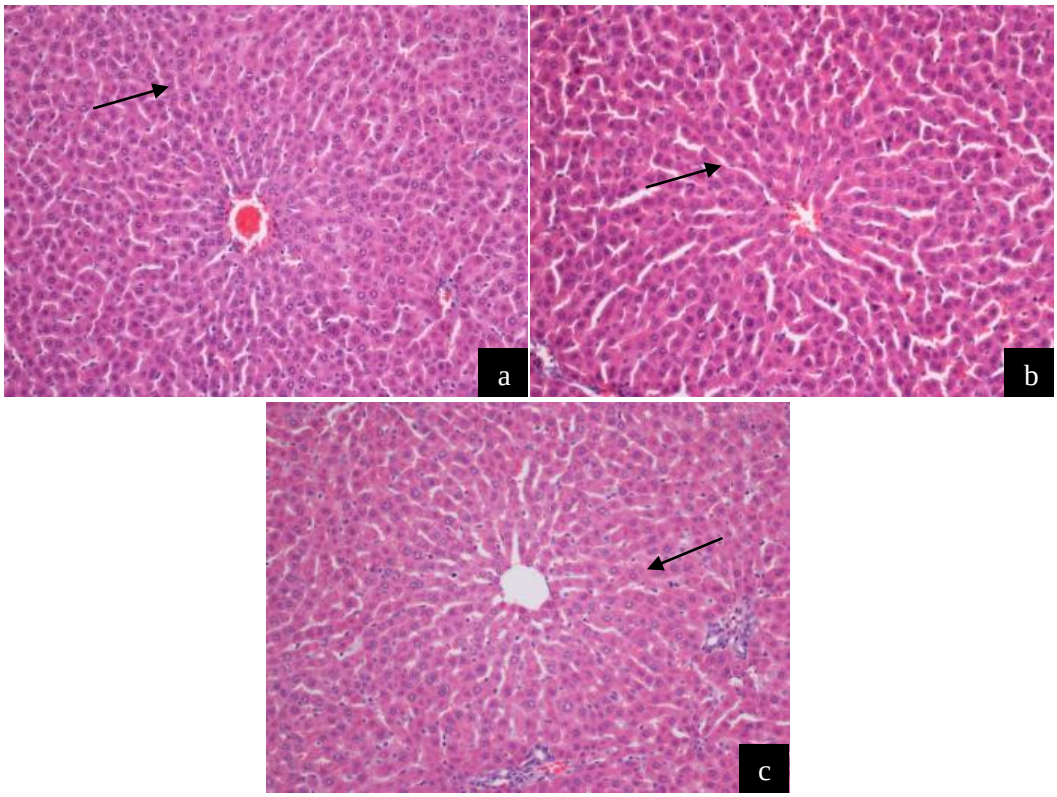

Plate. 4.1: Histological sections of liver in acute toxicity test. (a) Rats treated with 5 ml/kg vehicle (distilled water), (b) Rats treated with 2 g/kg (5 ml/kg) *H. erinaceus* extract, (c) Rats treated with 5 g/kg (5 ml/kg) *H. erinaceus* extract. (Haematoxylin and Eosin staining, 20x). Note the hepatocytes were arranged in cords (arrows).

The kidney of rats that were oral-administered with low and high doses of extracts showed normal glomeruli with normal vascularity. The collecting tubules were similar to that of control groups (Plate 4.2 a-c). The epithelial linings of both proximal and distal tubules were of normal architecture. No signs of morphological injury or

lesion were found in the kidney. These results indicated that the *H. erinaceus* extract did not have toxic effects on the physiological activities of the kidney.

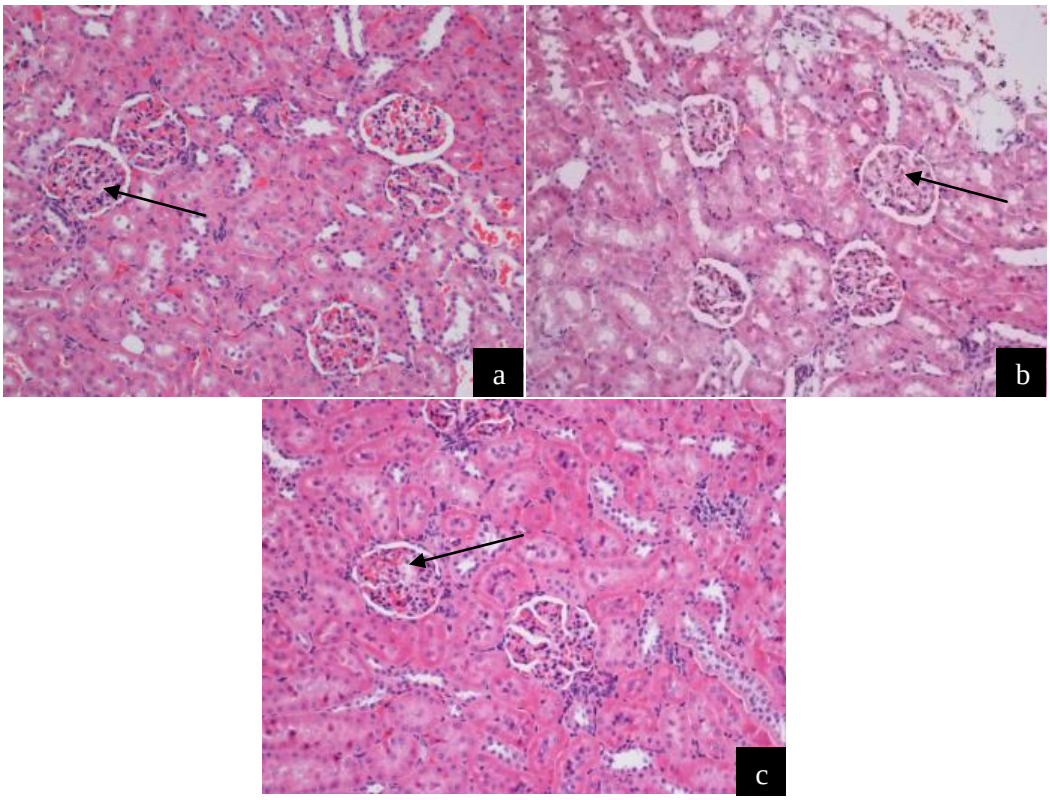

Plate 4.2: Histological sections of kidney in acute toxicity test. (a) Rats treated with 5 ml/kg vehicle (distilled water), (b) Rats treated with 2 g/kg (5 ml/kg) *H. erinaceus* extract, (c) Rats treated with 5 g/kg (5 ml/kg) *H. erinaceus* extract. (Haematoxylin and Eosin staining, 20x). Note the normal glomeruli (arrows).

4.1.3 Hematological and clinical biochemistry

The effects of aqueous extracts of *H. erinaceus* on haematological parameters of treated and control rats are presented in Table 4.1. The hematological status of rats after 14 days of oral-administration of *H. erinaceus* extract was not significantly ($p < 0.05$) different when compared to the control group rats. Both animals and humans have similar toxicity patterns in haematological, gastrointestinal and cardiovascular adverse

effects (Oslon *et al*., 2000). Hematological parameters in animals are important markers to reveal the risk of toxic as the changes in the blood system have a higher predictive value for human toxicity. In this study, Table 4.1 shows that both sexes of rats had increased level of Erythrocyte (RBC) in extracts group when compared to control group, though there were no significant ($p < 0.05$) difference between the three groups. The leukocyte (WBC) level in female high dose rats, 5.45 ± 0.56 (10^9/L) was higher than in control group 4.47 ± 0.55 (10^9/L), though no significant difference were found between groups. Further, in female rats, there was an increase trend for platelet parameter in extracts group when compared to control group. Platelet level was 691.00 ± 56.42 (10^9/L) in low dose group and 745.50 ± 33.35 (10^9/L) in high dose group. Both groups showed higher level than rats in control group (640.33 ± 43.64 (10^9/L)). However, there were no significant ($p < 0.05$) difference between the three groups. Erythrocyte (RBC), leukocyte (WBC), or thrombocytes (platelet) are blood cells formed during hematopoiesis process. Increased production in RBC, WBC and platelet indicated that there was a stimulation of the hematopoiesis system (Guyton & Hall, 2006). Erythrocyte plays role in boosting the immune system, WBC defend our body from being attack by foreign organisms and the platelets protect blood vessels from endothelial damage as well as initiate repair of these vessels. Thus, this suggests that *H. erinaceus* extract may have strong immuno-modulatory, antioxidant and endothelial protection and repair activity. As shown in Table 4.1, there were no significant changes ($p < 0.05$) in MCV, MCH, MCHC and RDW. Moreno Chulilla *et al.* (2009) stated that these parameters are major indices for the basis of morphological anaemia classification. In the present study, the extract did not significantly change the calculated RBC indices and had minimal effect on the size of RBC and in Hb content per RBC. This shows that *H. erinaceus* extracts may not have the potential to induce anaemia.

Table 4.1: Hematological parameters of the male and female rats treated with distilled water and *H. erinaceus* aqueous extract during the acute toxicity study.

	Control group		Low dose group		High dose group	
	Male	Female	Male	Female	Male	Female
HGB (g/L)	147.33 ± 3.12	141.00 ± 3.40	150.67 ± 2.89	132.00 ± 9.01	145.00 ± 2.50	144.17 ± 1.90
HCT (L/L)	0.48 ± 0.01	0.46 ± 0.01	0.49 ± 0.01	0.46 ± 0.01	0.48 ± 0.01	0.48 ± 0.01
RBC (10^12/L)	8.18 ± 0.13	7.80 ± 0.24	8.40 ± 0.24	7.84 ± 0.21	8.39 ± 0.14	7.98 ± 0.16
MCV (fl)	58.50 ± 1.38	59.50 ± 1.06	58.00 ± 1.03	58.50 ± 0.76	56.83 ± 0.31	59.67 ± 0.95
MCH (pg)	18.03 ± 0.37	18.08 ± 0.25	18.00 ± 0.29	16.78 ± 0.92	17.27 ± 0.13	18.08 ± 0.23
MCHC (g/L)	308.50 ± 2.57	304.83 ± 3.20	310.17 ± 0.70	287.83 ± 16.21	304.17 ± 1.54	304.00 ± 1.79
RDW (%)	15.70 ± 0.58	15.05 ± 0.49	14.77 ± 0.45	13.85 ± 0.25	16.02 ± 0.46	14.00 ± 0.28
WBC (10^9/L)	6.32 ± 0.72	4.47 ± 0.55	7.48 ± 0.44	4.42 ± 0.50	6.28 ± 0.47	5.45 ± 0.56
Platelet (10^9/L)	759.33 ± 27.88	640.33 ± 43.64	738.00 ± 50.79	691.00 ± 56.42	756.67 ± 30.26	745.50 ± 33.35

HGB: Hemoglolin;

HCT: Hematocrit;

RBC: Red Blood Cell (eErythrocyte);

MCV: Mean Corpuscular Volume;

MCH: Mean Corpuscular Hemoglolin;

MCHC: Mean Corpuscular Hemoglolin;

RDW: Red Blood Cell Distribution Width;

WBC: White Blood Cell (leukocyte)

All values were expressed as mean and ± standard error mean. No significant differences in treated rats when compared with control rats.

The biochemical analyses were assessed in both female and male rats and summarised in the Table 4.2 and 4.3. In renal function tests, there were no significant differences between treated female rats and control rats. Further, renal profiles of male rats were not significantly different when compared to the control except for potassium level. Potassium level was significantly ($p < 0.05$) elevated when compared to the rats in control group (4.77 ± 0.06 mmol/L). In the low dose group, the level was 5.08 ± 0.08 mmol/L whereas in the high dose group the level was 5.08 ± 0.09 mmol/L, and there was no significant difference ($p < 0.05$) between the two groups (Table 4.2).

Normal renal function is related to normal concentration of potassium in serum because more than 80% of the ingested potassium is excreted in the urine (Van Y Persele De Strihou, 1977). Serum potassium level which showed slightly above normal limits is a progressive sign of renal failure. However, normal architecture that was observed in kidney (Plate 4.2 b and c) showed that *H. erinaceus* extracts did not affect the kidney function when compared to normal group rats.

The observation of differences in renal function could be linked with the chronic progressive nephropathy that may affect the Sprague-Dawley rats. Kohn and Clifford (2002) reported that this age-related disease is more frequent in male rats. Stock animal variations, however, may be the main factor that caused the differences in elevated potassium levels in both groups of rats treated with low and high doses of extract.

From the renal function profile result, creatinine and urea levels in extracts group was not significantly different ($p < 0.05$) when compared to the control rats. Creatinine and urea are two important markers of kidney function (Arneson & Brickell, 2007). The insignificant changes in creatinine and urea levels implied that the activity of

protein metabolism was maintained within the normal range due to the non-toxic effect of the extract.

Table 4.2: Renal function parameters of the male and female rats treated with distilled water and *H. erinaceus* aqueous extract during the acute toxicity study.

	Control group		Low dose group		High dose group	
	Male	Female	Male	Female	Male	Female
Sodium (mmol/L)	142.00 ± 0.58	141.83 ± 0.40	142.00 ± 0.52	141.00 ± 0.68	142.50 ± 0.34	141.17 ± 0.48
Potassium (mmol/L)	4.77 ± 0.06[a]	4.62 ± 0.12	5.08 ± 0.08[b]	4.57 ± 0.16	5.08 ± 0.09[b]	4.53 ± 0.08
Chloride (mmol/L)	104.00 ± 0.82	105.33 ± 0.67	106.33 ± 1.02	105.33 ± 0.67	105.17 ± 0.54	106.00 ± 0.63
Carbon Dioxide (mmol/L)	24.82 ± 0.54	23.33 ± 0.41	22.60 ± 1.18	22.95 ± 0.41	23.92 ± 1.03	21.95 ± 0.77
Anion Gap (mmol/L)	18.17 ± 0.48	18.00 ± 0.26	18.17 ± 0.60	17.33 ± 0.49	18.50 ± 0.43	17.67 ± 0.49
Urea (mmol/L)	5.20 ± 0.35	7.93 ± 0.31	6.20 ± 0.50	7.97 ± 0.19	5.63 ± 0.18	8.30 ± 0.67
Creatinine (µmol/L)	34.83 ± 2.27	41.00 ± 2.79	33.67 ± 2.69	42.00 ± 4.76	34.33 ± 4.96	41.33 ± 2.12

All values were expressed as mean and ± standard error mean. Means with different superscripts were significantly different ($p < 0.05$) by Tukey Test. Six replicates of animals were used in each group.

For the evaluation of liver function, as shown in Table 4.3, male rats showed significant difference ($p < 0.05$) in alanine aminotransferase (ALT). Control group had the highest ALT value (51.83 ± 1.92 IU/L) when compared to low dose group (48.00 ± 0.58 IU/L) and high dose group (46.50 ± 1.45 IU/L) whereas in female rats, there was significant difference ($p < 0.05$) in alkaline phosphatase (ALP) compared control group (114.83±10.10 IU/L) and low dose group (72.83 ± 5.16 IU/L) (Table 4.3). From the result, male rats treated with low dose extract had higher ALP level (163.17 ± 15.40 IU/L) than control group (153.00 ± 15.14 IU/L), followed by high dose group (135.00 ± 9.04 IU/L). However, there was no significant difference ($p < 0.05$) between the three groups. The variation of liver linked enzymatic activity could be associated of age, sex and other factors (i.e. diets) (Braun *et al*., 1993). However, the ALP variation is well related to the (6-8 weeks) age of animals and especially with the higher bone metabolism in younger ones.

Table 4.3 shows that both sexes of rats showed increased trend in AST level in *H. erinaceus* extract groups when compared to control group. However, there was no significant difference ($p < 0.05$) between the groups. Both sexes of rats had the lowest AST level in control group. In male rats, low dose group had the highest AST level (181.83 ± 5.48 IU/L), followed by high dose group (175.83 ± 8.51 IU/L). Female rats in low dose group had 172.17 ± 10.35 IU/L, whereby the level of AST in high dose group increased by 10 IU/L, which is 182.83 ± 5.61 IU/L.

As shown in Table 4.3, male control rats had same G-Glutamyl Transferase level with male rats treated with high dose of extract (3.17 ± 0.17 IU/L), and both groups showed lower level than low dose groups (3.67 ± 0.42 IU/L). However, all three groups showed no significant difference ($p < 0.05$). In contrast to male group, female control group had the highest G-Glutamyl Transferase level (3.67 ± 0.33 IU/L), followed by low dose group (3.50 ± 0.50 IU/L) and high dose group (3.00 ± 0.00 IU/L).

Both groups did not show significant difference though there were changes in the groups.

Table 4.3: Liver function parameters of the male and female rats treated with distilled water and *H. erinaceus* aqueous extract during the acute toxicity study.

	Control group		Low dose group		High dose group	
	Male	Female	Male	Female	Male	Female
Total Protein (g/L)	61.33 ± 1.15	64.33 ± 1.26	57.83 ± 0.65	63.33 ± 1.17	60.17 ± 1.01	65.00 ± 0.68
Albumin (g/L)	9.83 ± 0.48	11.17 ± 0.17	8.67 ± 0.33	11.00 ± 0.45	9.83 ± 0.48	11.50 ± 0.43
Globulin (g/L)	51.50 ± 1.09	53.17 ± 1.28	49.17 ± 0.60	52.33 ± 1.26	50.33 ± 1.09	53.50 ± 0.72
Total Bilirubin (µmol/L)	2.17 ± 0.17	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00
Conjugated Bilirubin (µmol/L)	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
Alkaline Phosphatase (IU/L)	153.00 ± 15.14	114.83 ± 10.10^{b}	163.17 ± 15.40	72.83 ± 5.16^{a}	135.00 ± 9.04	94.00 ± 7.15^{ab}
Alanine Aminotransferase (IU/L)	51.83 ± 1.92^{b}	43.17 ± 1.94	48.00 ± 0.58^{ab}	39.67 ± 2.70	46.50 ± 1.45^{a}	44.50 ± 1.88
AST (IU/L)	171.50 ± 7.13	171.33 ± 6.84	181.83 ± 5.48	172.17 ± 10.35	175.83 ± 8.51	182.83 ± 5.61
G-Glutamyl Transferase (IU/L)	3.17 ± 0.17	3.67 ± 0.33	3.67 ± 0.42	3.50 ± 0.50	3.17 ± 0.17	3.00 ± 0.00

All values were expressed as mean and ± standard error mean. Means with different superscripts were significantly different ($p < 0.05$) by Tukey Test. Six replicates of animals were used in each group.

Among the biochemical parameters tested, AST, ALT and ALP are considered as important markers of liver function (Arneson & Brickell, 2007; and Obici *et al.*, 2008). Rise of AST and ALT level in serum level are indicators for hepatocellular damage. Aniagu *et al.* (2004) stated that ALT enzyme is considered a more sensitive marker of hepatocellular damage than AST because ALT is localized in the cytosol of hepatocytes and provides a quantitative evaluation of the degree of damage sustained by the liver.

Increase in ALP level is often linked with various disorders such as extra-hepatic bile obstruction, intra-hepatic cholestasis, infiltrative liver disease, hepatitis and bone diseases (Adinortey *et al.*, 2012). However, increase of ALP in less than three times the normal level is considered non-specific and will not affect the normal activity of liver (Rosalki & Mcintyre, 1999; and Pagana & Pagana, 2003). Mukherjee (2005) demonstrated that extremely high ALP level in serum is seen in cases of obstructive jaundice and biliary cirrhosis. Both ALP and GGT levels are expected to be high if there is bile duct obstruction in liver. Since there were no significant changes ($p < 0.05$) in both ALP and GGT levels, this indicated that normal liver function was observed after administration of *H. erinaceus* extract. This result correlated with findings from histopathological examination of the liver, as it showed normal cellular architecture.

Bilirubin is a major breakdown product of haemoglobin. Therefore, bilirubin level in serum is considered an indicator of liver function because it shows that the liver possesses ability to take up, process and secrete bilirubin into the bile (Abotsi *et al.*, 2011). Table 4.3 reveals that both sexes of rats showed no significant difference ($p < 0.05$) and change in total bilirubin and conjugated bilirubin between control and extract groups. From the results, *H. erinaceus* extract did not show any adverse effects on hepatic metabolism or bililary secretion.

Plasma proteins such as total protein, albumin and globulin are produced by liver. After 14 days of *H. erinaceus* extract administration, the level of total protein, albumin and globulin in extract treated groups were not significantly different ($p < 0.05$) when compared to the control group. Therefore, administration of *H. erinaceus* extracts did not affect the production of plasma protein in liver.

In the present study, the *H. erinaceus* extract did not affect the clinical biochemistry parameters and body weight. Further, histological evaluation showed no signs of toxicity. Therefore, the extract was considered safe at concentrations tested. There were no signs of acute toxicity and the oral lethal dose (LD_{50}) for the male and female rats was greater than 5 g/kg body weight.

4.2 Gastroprotective effects of *H. erinaceus* extract *in vivo*

4.2.1 Effect of *H. erinaceus* extracts on gross ethanol-induced gastric lesions in rats

The gastroprotective activity of *H. erinaceus* extract in ethanol-induced gastric lesion model is shown in Plate 4.3 (a-g). It was observed that the gastroprotective effect of *H. erinaceus* extracts on gastric mucosa was in a dose dependent manner (Plate 4.3a-g). Ethanol produced extensive visible black hemorrhagic lesions of gastric mucosa (Plate 4.3b). Results showed that rats that were oral-administered with omeprazole or *H. erinaceus* extracts had significantly ($p < 0.05$) reduced areas of gastric ulcer formation as compared with ulcer control group (Plate 4.3b). In addition, 400 mg/kg of *H. erinaceus* extract significantly ($p < 0.05$) inhibited the formation of the ulcers. It was observed that protection of gastric mucosa was the most significant ($p < 0.05$) in rats pre-treated with 400 mg/kg mushroom extract (Plate 4.3g).

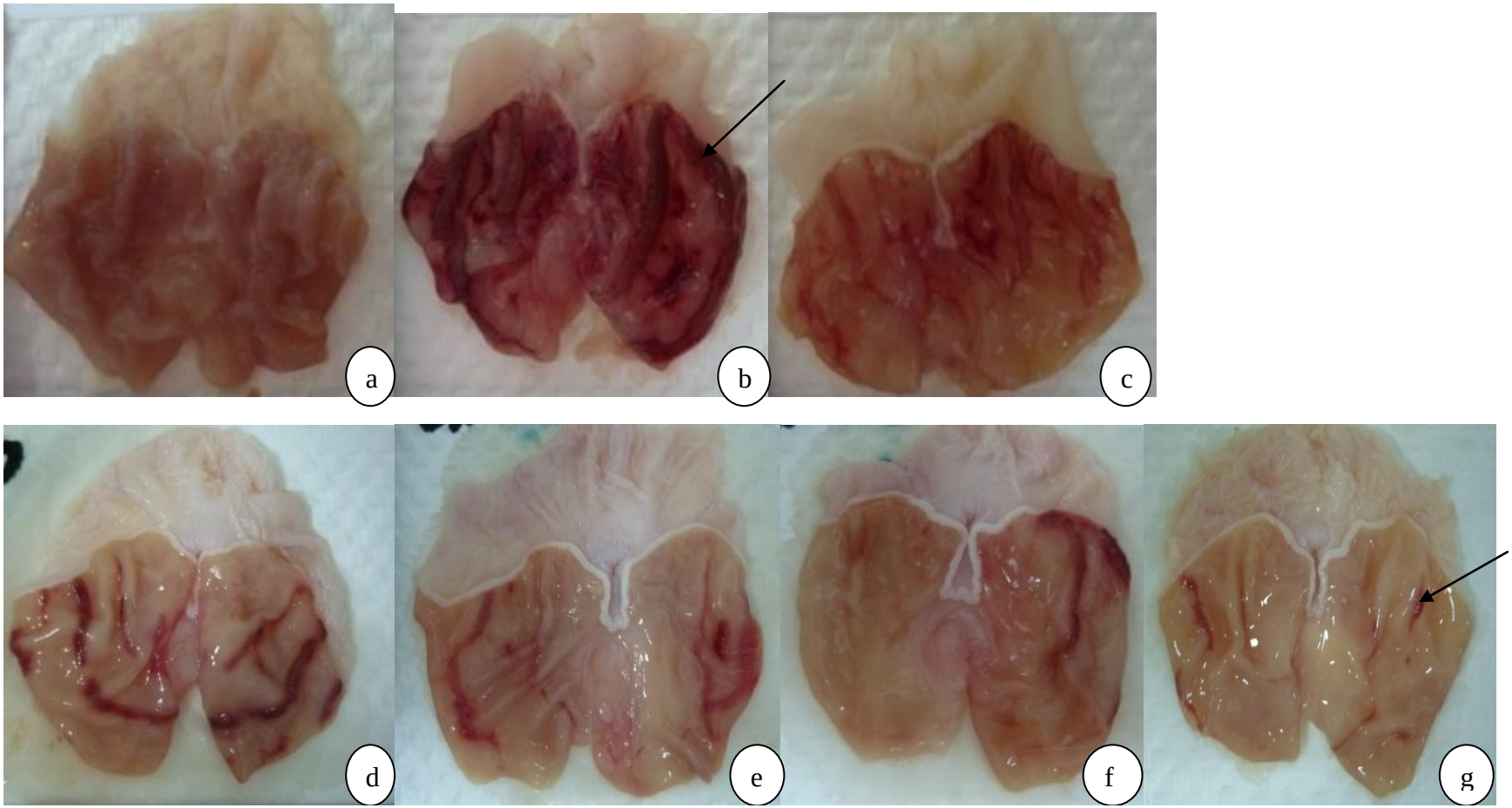

Plate 4.3: Gross appearance of the gastric mucosa in rats with ethanol-induced ulcers. (a) Rats pre-treated with 5ml/kg distilled water (normal control). No injuries to the gastric mucosa are observed. (b) Rats pre-treated with 5ml/kg distilled water (ulcer control). Severe injuries are observed in the gastric mucosa. Ethanol produced extensive visible hemorrhagic necrosis (arrow) of gastric mucosa. (c) Rats pre-treated with omeprazole (20mg/kg). Injuries to the gastric mucosa are milder compared to the injuries seen in the ulcer control rats. (d). Rats pre-treated with *H. erinaceus* extract (50 mg/kg). Moderate injuries are seen in the gastric mucosa. The extract inhibits the formation of gastric lesions induced by ethanol. (e). Rats pre-treated with *H. erinaceus* extract (100 mg/kg).Moderate injuries are seen in the gastric mucosa. The extract inhibits the formation of gastric lesions induced by ethanol (f). Rats pre-treated with *H. erinaceus* extract (200 mg/kg). Mild injuries are seen in the gastric mucosa. The extract reduces the formation of gastric lesions induced by ethanol. (g) Rats pre-treated with 400 mg/kg of *H. erinaceus* extract. Very mild injuries (arrow) to the gastric mucosa are seen.

4.2.2 Effect of *H. erinaceus* on ulcer area and ulcer inhibition in gastric glandular tissue of rats

Ulcer in gastric glandular tissue of pre-treatment ulcer control group (distilled water) showed severe mucosal injury (Figure 4.3), whereas the rats pre-treated with omeprazole (20mg/kg) or *H. erinaceus* extract (50, 100, 200, and 400mg/kg) showed significantly ($p < 0.05$) decreased area of gastric lesion formation compared to pre-treatment with ulcer control group. *Hericium erinaceus* extract significantly prevented the formation of ulcer, in a dose-dependant manner. Ulcer control group had the largest ulcer area (894.00 ± 36.69 mm^2), rats that were treated with *H. erinaceus* extract at a dose of 50mg/kg had 727.20 ± 26.88 mm^2 of ulcer area, followed by 602.40 ± 29.43 mm^2 with 100mg/kg of *H. erinaceus* extract. The inhibition of ulcer was significant ($p < 0.05$) when compared to the standard drug group. There was, however, no significant difference ($p < 0.05$) between the highest dosage of extract (240.00 ± 17.31 mm^2), 400mg/kg and the standard drug omeprazole group (285.6 mm^2). Ulcer areas in rats treated with 50mg/kg of *H. erinaceus* extract (727.20 ± 26.88 mm^2) were three times larger than rats treated with 400mg/kg *H. erinaceus* extract (240.00 ± 17.31 mm^2). Higher doses of extract gave better protection against the formation of the ulcers induced by ethanol.

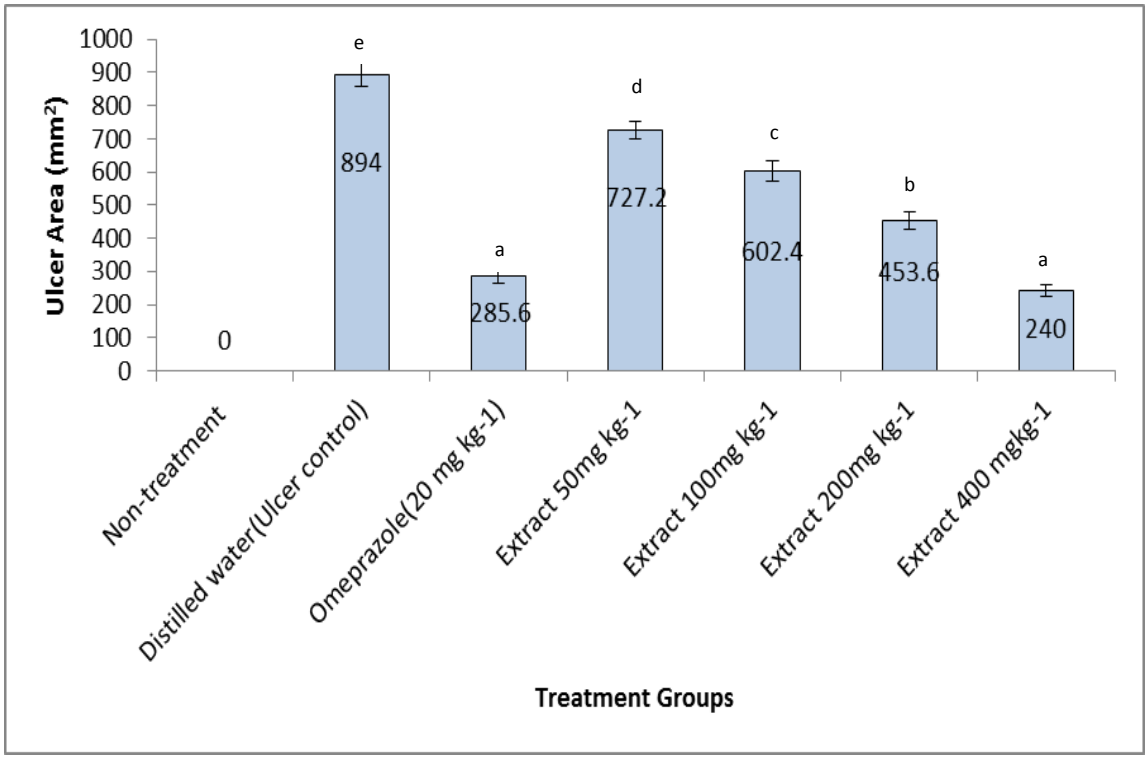

Figure 4.3: Effect of *H. erinaceus* extract on ulcer area in ethanol induced ulcers in rats. All values were expressed as mean of six replicate values. Means with different letters were significantly different ($p < 0.05$) by Tukey Test. Six replicates animals were in each treatment group.

Figure 4.4 showed the ulcer inhibition percentage for seven different groups of treatment. As shown in Figure 4.4, inhibition percentage of ulcer of *H. erinaceus* extracts was increased in a dose dependent manner. Pre-treatment with *H. erinaceus* extract (400mg/kg) exhibited the highest inhibition percentage of ulcer area formation, at 72.97%, followed by omeprazole (67.45%), 200mg/kg (48.6%), 100mg/kg (32.17%) and 50mg/kg (18.14%). Inhibition percentage of ulcer in omeprazole group (67.45%) was comparable to 400mg/kg extract group (72.97%).

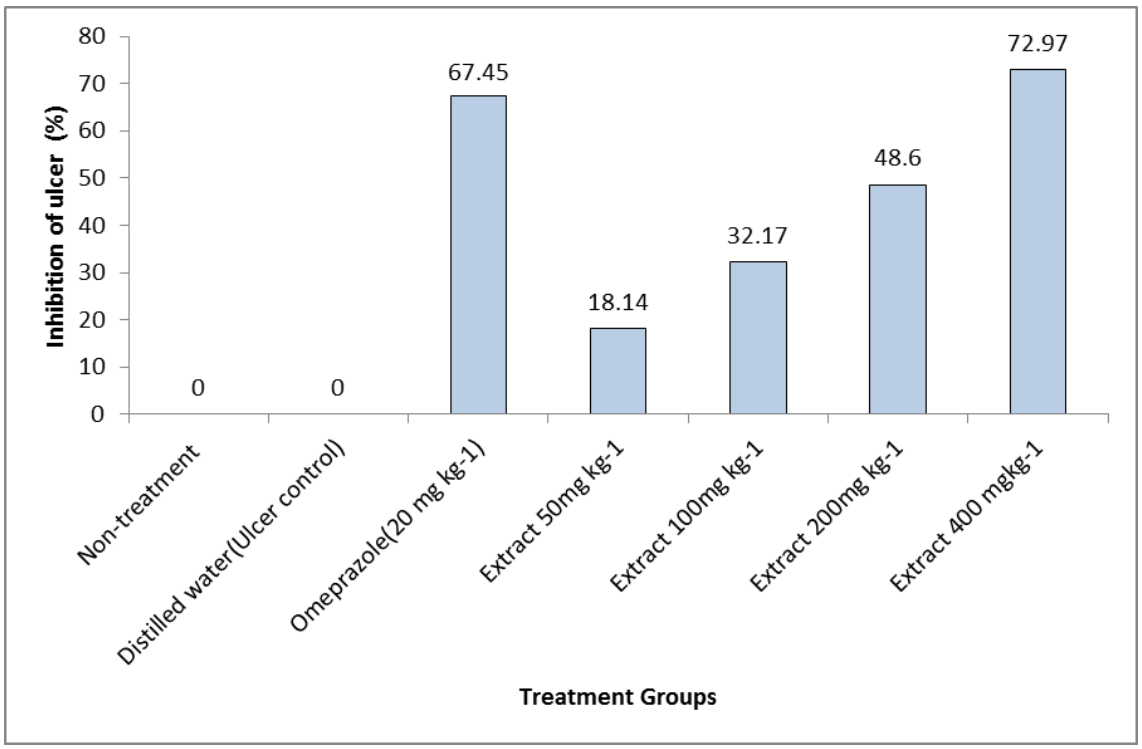

Figure 4.4: Inhibitory (%) effect of *H. erinaceus* extract on ethanol-induced ulcer in rats. All values were expressed as mean of six replicate values. Means with different letters were significantly different ($p < 0.05$) by Tukey Test. Six animals were used in each treatment group.

Ethanol-induced gastric ulcer has been widely used for the experimental evaluation of anti-ulcer activity. Gastric lesions produced by ethanol administration appeared as multiple haemorrhagic red bands of different sizes along the glandular stomach. Szabo *et al*. (1995) suggest that the ethanol damage to the gastrointestinal mucosa starts with microvascular injury, namely disruption of the vascular endothelium resulting in increased vascular permeability, edema formation and epithelial lifting. Ethanol produces necrotic lesions in the gastric mucosa by its direct toxic effect, reducing the secretion of bicarbonates and production of mucus (Marhuenda *et al*., 1993). Exposure to ethanol increases the extension of cellular damage in a dose-dependent way (Mutoh *et al*., 1990). The *H. erinaceus* extract at a dose of 400 mg/kg gave a 72.93% of ulcer inhibition of the gastric mucosa. This indicated that this mushroom possessed protective action on the gastric mucosa (Figure 4.4). This result also suggested a possible cytoprotective activity, since ethanol acts directly on gastric

mucosal cells. It is well known that ethanol induced gastric ulcers are not inhibited by anti-secretory agents such as cimetidine. Thus, omeprazole, one of the proton pump inhibitors, was being used in current study as a control.

4.2.3 Effect of *H. erinaceus* on mucus production in gastric glandular tissue of rats

The mucus weight of the stomach was measured in all test groups of rats, the mucus content (g) in the positive control group, administered with omeprazole had a higher mucus weight of 1.56 ± 0.07 g as compared to all treated rats (Figure 4.5). Rats that were orally administered with ethanol had significantly ($p < 0.05$) lower mucus weight (0.79 ± 0.03 g) when compared to rats in positive group. However, there was no significant ($p < 0.05$) difference between groups treated with *H. erinaceus* extract at concentration of 50, 100, and 200 mg/kg (Figure 4.5).

Rats in non-treated group that were only administered with distilled water had significantly ($p < 0.05$) lower mucus weight (0.43 ± 0.06 g) as compared to animals with ulcer induced by ethanol. This suggested that tissue damage in the stomach would increase the production of mucus weight. To regain the balance, drugs of plant origin were investigated to inhibit the gastric acid secretion or to activate the mucosal defence mechanism by increasing mucus production (Borrelli & Izzo, 2000). The results of this study have revealed that the *H. erinaceus* extracts were able to stimulate the increase amount of mucus that can protect stomach against the formation of ulcer induced by ethanol.

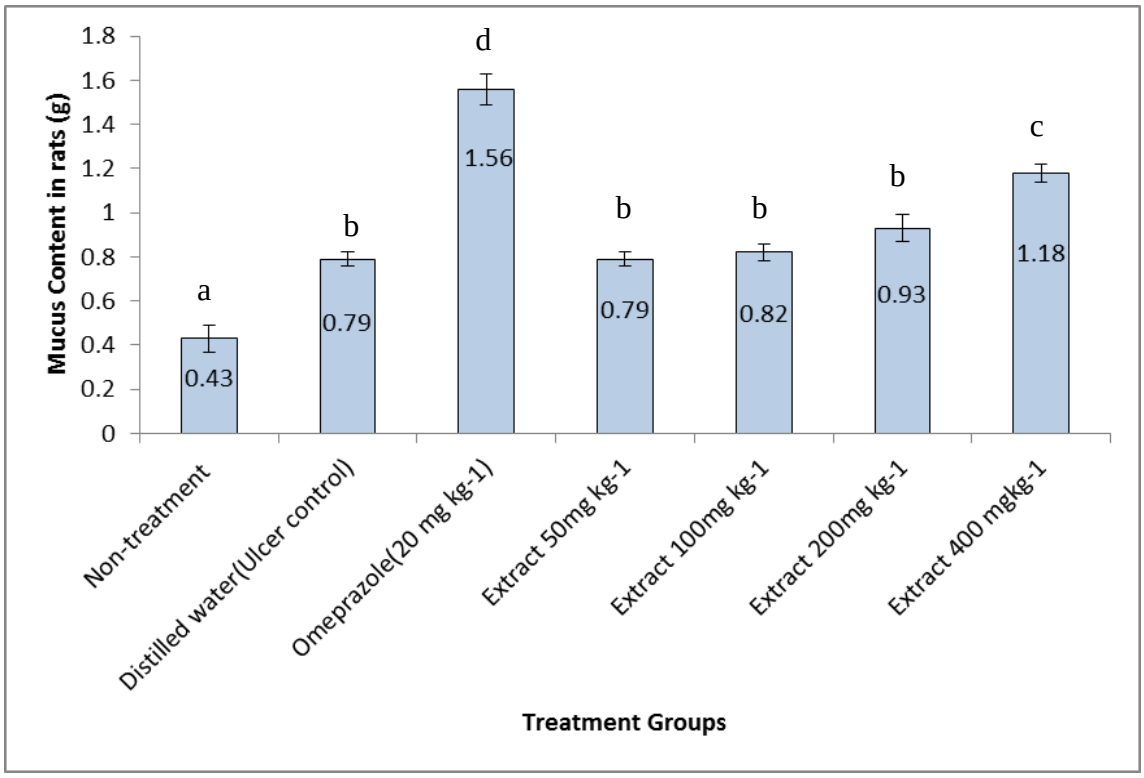

Figure 4.5: Effect of *H. erinaceus* extract on mucus production in rats with ethanol induced ulcers. All values were expressed as mean of six replicate values. Means with different letters were significantly different ($p < 0.05$) by Tukey Test. Six animals were in each treatment group.

It was demonstrated that ulceration induced by ethanol is associated with diminished protective factors of the mucosa such as prostaglandin and mucus (Konturek *et al.*, 2005). In light of this, the gastroprotective activity of *H. erinaceus* may be attributed to the mucosal protective factor as mucus production increased with increasing concentration of extracts. Davenport (1968) reported that the mucus of the gastric wall is thought to play an important role as a defensive factor against gastrointestinal damage. From our results, pre-treatment with *H. erinaceus* aqueous extract significantly ($p < 0.05$) increased the gastric mucus production (Figure 4.5). This showed that *H. erinaceus* aqueous extract may play role in the preservation of gastric mucus secretion. Mucus is a viscous, elastic, adherent and transparent gel comprised of 95% water and 5% glycoprotein. Glycoprotein present in mucus is an important

protective factor for the gastric mucosa because glycoprotein functions as antioxidant, reducing damage in the mucosa induced by free radicals (Repetto & Llesuy, 2002).

4.2.4 Effect of *H. erinaceus* in gastric barrier mucus production in gastric glandular tissue of rats with ethanol-induced ulcer

The effect of distilled water (5 ml/kg), omeprazole (20 mg/kg), and different dosage of *H. erinaceus* extracts (50, 100,200, 400 mg/kg) on gastric barrier mucus in gastric ulcer rats induced by ethanol are shown in Figure 4.6. The alcian blue binding capacity in ulcer control group (3.42 ± 0.13 μg/g) was siginificantly ($p < 0.05$) lower than in the non-treated rats (5.01 ± 0.46 μg/g) and rats pre-treated with omeprazole and *H. erinaceus* extracts. Omeprazole treated group (6.73 ± 0.67 μg/g) had a significant lower amount of gastric mucus as compared to *H. erinaceus* extract groups. It was observed that the amount of gastric mucus in rats treated by *H. erinaceus* extracts groups was increased in a dose dependent manner. The binding capacity, however, decreased at dosage 400 mg/kg (10.64 ± 0.90 μg/g).

These results revealed that ethanol inhibited gastric mucus secretion. Similar result was reported in García-Barrantes and Badilla (2011). They had demonstrated that the binding capacity of alcian blue increased in a dose dependent manner in pylorus-ligated rats.

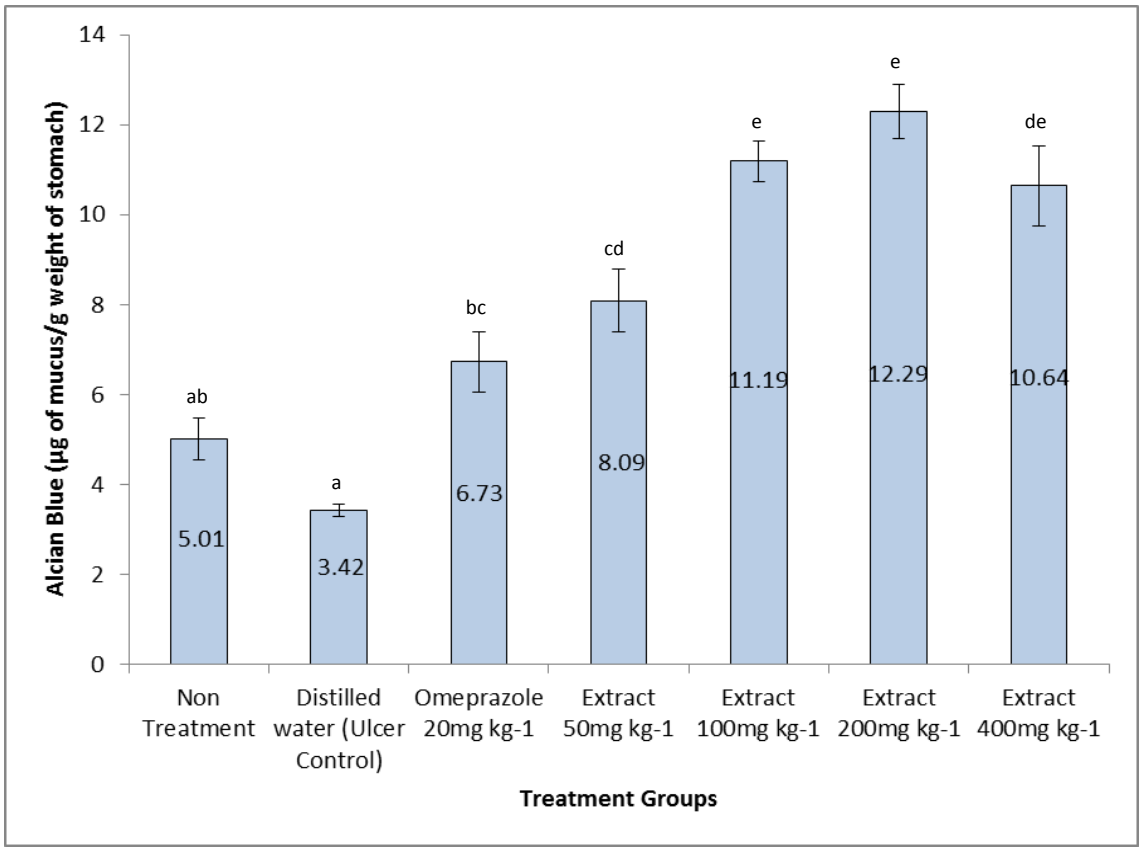

Figure 4.6: Effect of *H. erinaceus* extract on gastric barrier mucus in rats with ethanol induced ulcers. All values were expressed as mean of six replicate valus. Means with different letters were significantly different ($p < 0.05$) by Tukey Test. Six replicates animals were in each treatment group.

Gastric barrier mucus plays important role in gastroprotection (Kaunitz, 1999). Pre-treatment with *H. erinaceus* aqueous extract significantly ($p < 0.05$) increased the mucoprotective effect. The free mucus was significantly increased as compared to the mucus barrier of ulcer control animals ($p < 0.05$). This mucus comprises of mucin-type glycoprotein, which can be detected by a colour dye called alcian blue (Bolton *et al*., 1978). Alcian blue dye is able to bind to negatively charged materials. The mucus gel adhering to the gastric mucosal surface protects the underlying epithelium against acid, pepsin and necrotizing agents such as ethanol and indomethacin (Alqasoumi *et al*., 2008). In addition, Allen and Flemström (2005) demonstrated that gastric wall mucus plays crucial role in the defence of the gastric mucosa against aggression of chemical or mechanical factors compared to the soluble mucus in the lumen of the stomach. Gastric wall mucus coating may enhance the repair of the damaged gastric epithelium (Shih *et*

al., 2005). Hence, the increase of alcian blue binding capacity to gastric wall mucus suggested the involvement of *H. erinaceus* aqueous extract in process of mucosal adaptation by increasing the secretion of mucus in stomach of rats.

4.2.5 Histological evaluation of gastric lesions in stomachs of control and treated rats

Histological observation of ethanol induced gastric lesions in ulcer control group pre-treated with sterile distilled water (ulcer control group), showed comparatively extensive damage to the gastric mucosa and necrotic lesions penetrate deeply into mucosa, and extensive oedema and leucocytes infiltration of the submucosal layer were observed (Plate 4.4). Rats that were pre-treated with *H. erinaceus* extract had comparatively better protection of the gastric mucosa as seen by reduction or absence of ulcer area, reduced or absence of submucosal edema and leucocytes infiltration (Plate 4.4). *Hericium erinaceus* extract has been shown to have the cytoprotective effects in a dose-dependent manner. Previous studies that using mushroom extracts in gastroprotective did not evaluate on histological test.

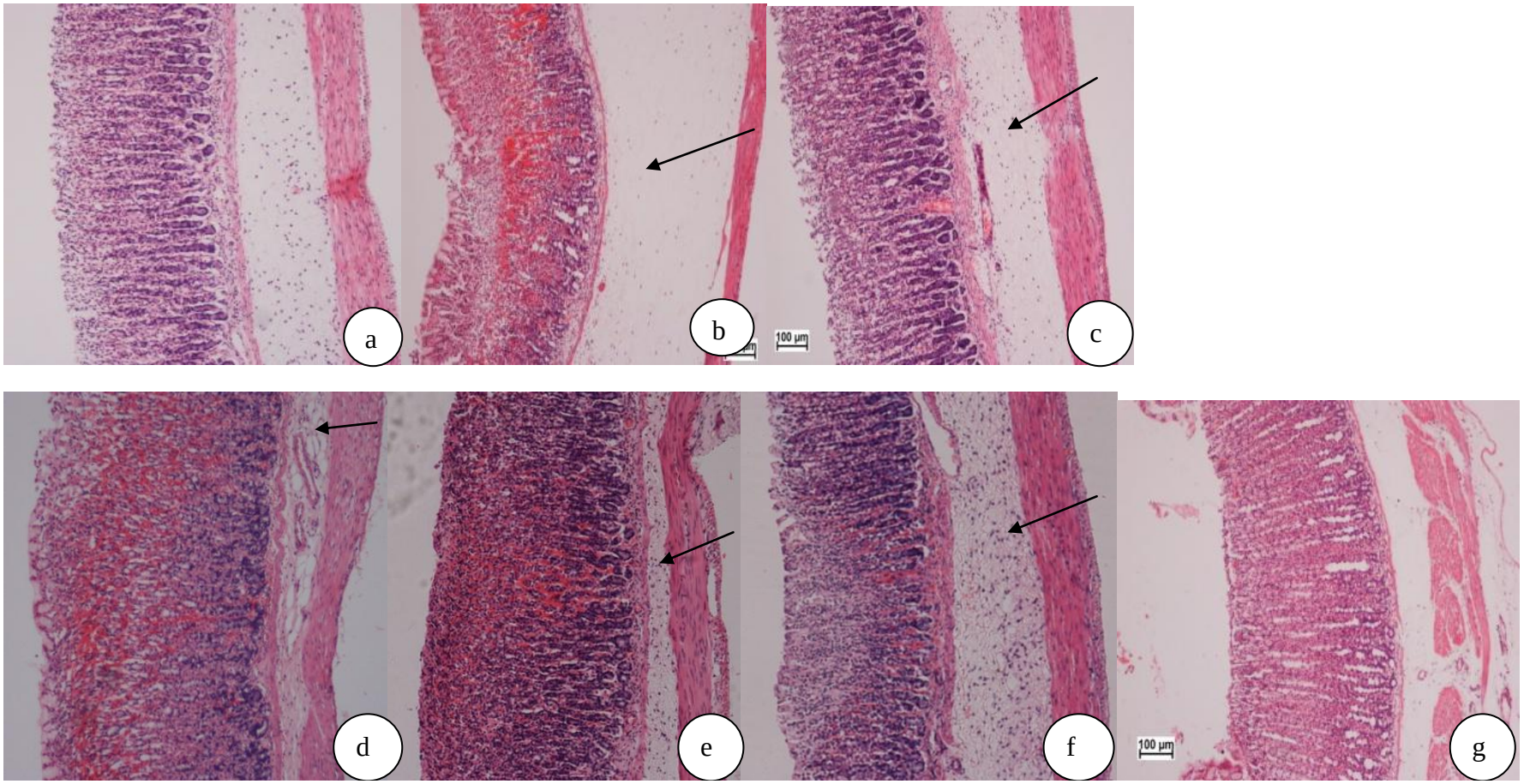

Plate 4.4: Histological study of ethanol-induced gastric mucosal damage in rats. (a) Rats pre-treated with 5ml/kg distilled water (normal control group). No injuries to the gastric mucosa are seen. (b) Rats pre-treated with 5ml/kg ethanol (ulcer control group). There is severe disruption to the surface epithelium and necrotic lesions penetrating deeply into mucosa and extensive edema of submucosa layer and leucocytes infiltration is present. (c) Rats pre-treated with omeprazole (20mg/kg). Mild disruption of the surface epithelium mucosa is seen. There is edema and leucocytes infiltration of the submucosal layer. (d) Rat pre-treated with *H. erinaceus* extract (50 mg/kg). Moderate disruption of surface epithelium is present. There is leucocytes infiltration in submucosal layer. (e) Rats pre-treated with *H. erinaceus* extract (100 mg/kg). There is mild disruption to the surface epithelium. There is edema with leucocytes infiltration of the submucosal layer. (f). Rats pre-treated with *H. erinaceus* extract (200 mg/kg). There is mild disruption to the surface epithelium. There is edema and leucocytes infiltration of the submucosal layer. (g) Rats pre-treated with *H. erinaceus* extract (400 mg/kg). There is no disruption to the surface epithelium and no edema or leucocytes infiltration of the submucosal layer (H&E stain 10× magnification). Notice leucocytes infiltration in submucosal layer (arrows).

Results of the present study showed protection of gastric mucosa and inhibition of leucocytes infiltration of gastric wall in rats pre-treated with *H. erinaceus* extract. Cheng and Koo (2000) demonstrated that pre-treatment with plant extract before ethanol administration significantly decreased neutrophil infiltration of gastric mucosa. While, Shimizu *et al*. (2000) had shown that the decrease in neutrophil infiltration into ulcerated gastric tissue promotes the healing of gastric ulcers in rats. Ethanol damaged the gastric mucosa and increased neutrophil infiltration into the gastric mucosa. Neutrophils play role in prevention of infection, destroy and remove bacteria as well as damage tissue by phagocytosis. Tsukimi *et al*. (1996) found that suppression of neutrophil infiltration during inflammation could enhance gastric ulcer healing. From the current study, *H. erinaceus* extract may possess anti-inflammatory activity too that protects against gastromucosa damage.

4.2.6 Immunohistochemistry analysis in the stomach of rats in ethanol-induced ulcer

Immunohistochemical results showed that rats pre-treated with *H. erinaceus* extract prior to ethanol administration expressed HSP70 protein in high level (Plate 4.5). In contrast, expression of HSP70 protein in ethanol-induced gastric tissues (ulcer control group) was found to be down regulated compared to HSP70 (70 kilodalton heat shock proteins) expression in *H. erinaceus* extract treated group (Plate 4.5). Immunohistochemical staining of Bax protein showed that pre-treatment of ethanol-induced ulcer with *H. erinaceus* extract caused down-regulation of Bax protein (Plate 4.5). Furthermore, up-regulation of Bax in ulcer control group was observed as compared to *H. erinaceus* extract-treated group (Plate 4.5).

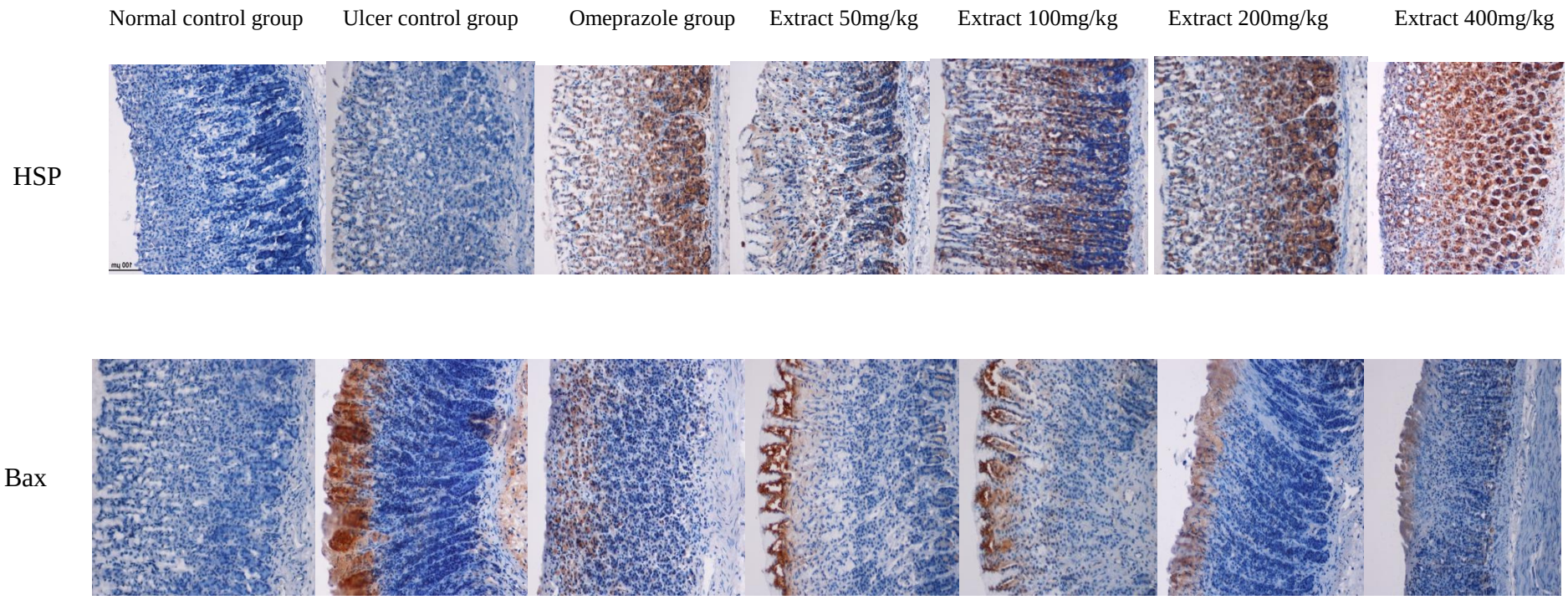

Plate 4.5: Immunohistochemical analysis of expression of HSP and Bax proteins in the stomach of rats in ethanol-induced gastric ulcer. Immunohistochemistry staining of HSP70 and Bax proteins showed up-regulation of HSP70 protein and down-regulation of Bax protein in rats pretreated with *H. erinaceus* extract (magnification 20×).

In the present study, there was up-regulation of heat shock protein 70 (HSP70) in the ulcerated mucosa of the group treated with *H. erinaceus* extract (Plate 4.5). This finding affirms previous studies which showed an increase in HSP70 expression after the damage of the gastric mucosa (Hirakawa *et al*., 1996; and Schichijo *et al*., 2003). Qiao *et al.* (2011) also demonstrated that Bax protein was expressed in gastric ischemia-reperfusion of rats. The level of Bax protein increased in early stage of reperfusion, reached its peak at one hour of reperfusion, and decreased gradually after that. Apoptosis and proliferation are basic mechanisms for cell death and survival and differentiation in the gastric mucosa. Up-regulation of Bax protein induced apoptosis and inhibited proliferation. Based on the results, extracts of *H. erinaceus* may have the ability to suppress and inhibit synthesis of Bax protein which may lead to the protection of the mushroom extract against irritants in stomach.

Heat shock proteins, (HSPs) play important roles in normal conditions and pathological situations involving both systemic and cellular stress. It have strong cytoprotective effects, are involved in many regulatory pathways, and act as molecular chaperones for preserving important cellular proteins (Hightower, 1991; and Arya *et al.*, 2007). In the gastric mucosa, it has been shown that HSP70 had important cytoprotective function in *in vitro* and *in vivo* (Nakamura *et al*., 1991; and Itoh & Noguchi, 2000). Our results show significant expression of HSP70 in pre-treated mushroom extract. The HSP70 family functions as a molecular chaperone and reduces stress-induced denaturation and aggregation of intracellular proteins. Reactive oxygen species (ROS) generated by ethanol suppresses the expression of HSP and increase the expression of Bax protein. HSP70 proteins protect cells from oxidative stress or heat shock. The over expression of HSP70 that was observed in this study could indicate that *H. erinaceus* extract protected the gastric tissues through the upregulation of HSP70.

4.2.7 Effect of *H. erinaceus* in LPO, SOD and CAT enzymes in gastric glandular tissue of rats with ethanol-induced ulcer

To determine the effects of antioxidant based defences on the ulceration process, the levels of antioxidants (SOD, CAT, and LPO) were evaluated in all stomach tissues. Ulcer control group had the highest LPO level (8.89 ± 0.98 μM TEP equivalent) among the treated groups (Figure 4.7). As the concentration of *H. erinaceus* extract was increased, the lipid peroxidation level had shown decreasing trend. *Hericium erinaceus* extract at a dose of 50 mg/kg had the highest LPO level (7.46 ± 0.15 μM TEP equivalent) among the mushroom extract groups, followed by 100mg/kg (6.83 ± 0.14 μM TEP equivalent) and 200mg/kg (6.02 ± 0.15 μM TEP equivalent). The result in 400mg/kg mushroom extract group (5.25 ± 0.07 μM TEP equivalent) showed no significant ($p < 0.05$) difference when compared to omeprazole group (5.44 ± 0.10 μM TEP equivalent). The administration of ethanol significantly ($p < 0.05$) increased the LPO level in the stomach tissues of rats in comparison to healthy rats.

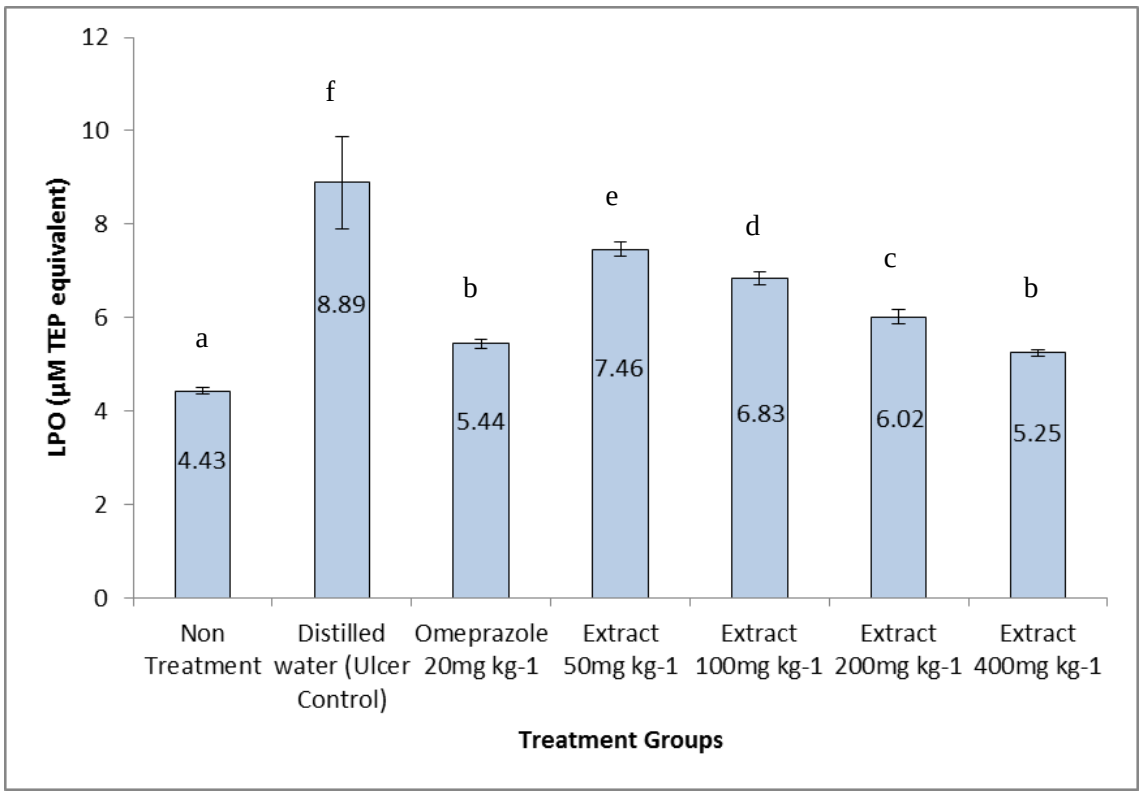

Figure 4.7: Effect of *H. erinaceus* extract on LPO production (µM TEP equivalent) in ethanol induced ulcer of rats. All values were expressed as mean of three replicate values. Means with different letters were significantly different ($p < 0.05$) by Tukey Test. Six replicates animals were in each treatment group.

Figure 4.8 shows production of SOD enzyme in gastric glandular tissue of rats with ethanol-induced ulcer. Ulcer control group produced the least amount of SOD enzyme (1.89 ± 0.16 U/mg), while *H. erinaceus* extract produced SOD enzyme in a dose dependent manner. *Hericium erinaceus* extract at a dose of 200mg/kg produced three fold of enzyme when compared to 50mg/kg extract. However, there was no significant ($p < 0.05$) difference of SOD enzyme production between omeprazole group (5.77 ± 0.20 U/mg) and extract at 200mg/kg (6.24 ± 0.39 U/mg).

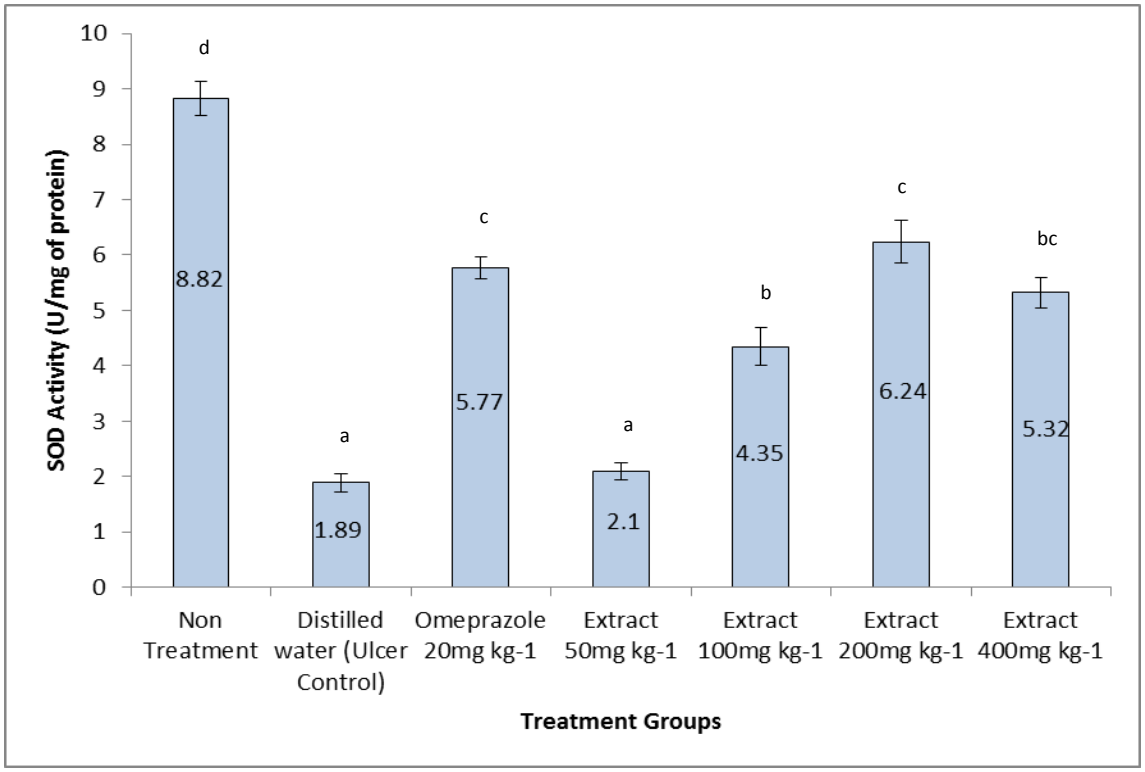

Figure 4.8: Effect of *H. erinaceus* extract on SOD production (U/mg of protein) in ethanol induced ulcer of rats. All values were expressed as mean of three replicate values. Means with different letters were significantly different ($P < 0.05$) by ANOVA Test. Six replicates animals were in each treatment group.

Catalase enzyme production in stomach of rats with ethanol-induced ulcer is shown in Figure 4.9. *Hericium erinaceus* extract at a dose of 50mg/kg showed the least production of catalase enzyme (40.45 ± 2.71 U/mg), whereas non-treatment group had the highest production of catalase enzyme (179.24 ± 11.89 U/mg). Mushroom extract produced catalase enzyme in a dose dependent manner, whereas extract at a dose of 200mg/kg produced the highest amount (135.94 ± 6.54 U/mg) of catalase enzyme among the mushroom extract groups. This was significantly higher ($p < 0.05$) when compared to rats treated with omeprazole and other doses of mushroom extracts.

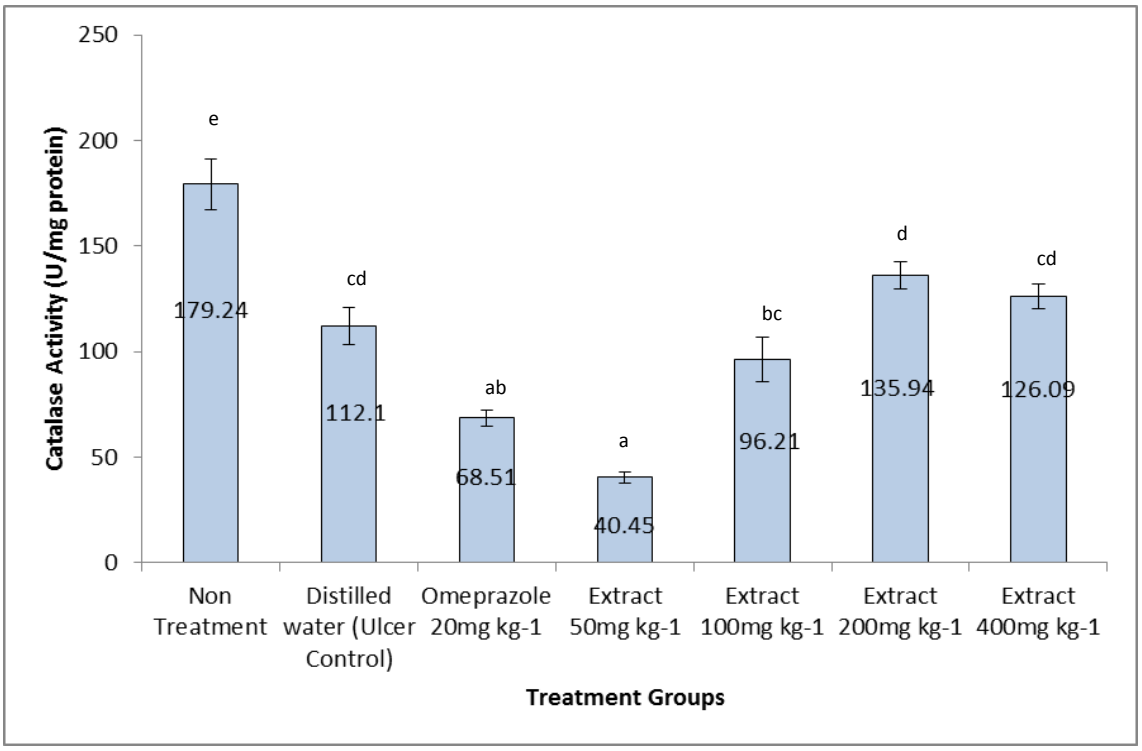

Figure 4.9: Effect of *H. erinaceus* extract on CAT production (U/mg protein) in ethanol induced ulcer of rats. All values were expressed as mean of three replicate values. Means with different letters were significantly different ($P < 0.05$) by ANOVA Test. Six replicates animals were in each treatment group.

Previous studies have shown that the administration of ethanol decreased the levels of SOD and CAT in tissues (Li *et al*., 2008; and Alimi *et al*., 2010). Similarly, in the present study, the levels of SOD and CAT in rat stomach tissues were significantly reduced by the administration of ethanol, and pretreatment with *H. erinaceus* extracts at doses of 200 and 400 mg/kg resulted in decrease on lipid peroxidation (Figure 4.7), and a significant increase in the levels of SOD and CAT (Figure 4.8 and Figure 4.9).

Reactive oxygen species are involved in the pathogenesis of ethanol-induced (Pihan *et al*., 1987) gastric mucosal injury *in vivo*. Arafa and Sayed-Ahmed (2003) found that the production of oxygen-free radicals plays a crucial role in the development of ethanol-induced gastric lesions. Huh *et al*. (2003) also revealed that free radical mechanisms contribute to ethanol-induced tissue injury. Superoxide produced by peroxidase in the stomach tissues might damage cell membranes and cause ulcer by increasing LPO level (Cadirci *et al*., 2007). Preventive anti-oxidants, such as superoxide

dismutase (SOD) and catalase (CAT) enzymes are the first line of defence against reactive oxygen species.

SOD scavenges the super oxide radical O_2^-, one of the reactive oxygen species (ROS) responsible for lipid peroxidation (Fridovich, 1978). This reaction leads to an increase in the generation of peroxyl radical $H_2O_2^-$, which is also capable of producing more oxidative damage (Das *et al*., 1997). Catalase and other peroxidases further reduce $H_2O_2^-$. In the present study, pre-treatment with *H. erinaceus* aqueous extract significantly decreased LPO and increased SOD and CAT, which indicates its efficacy in preventing free radical-induced damage.

A previous study had demonstrated that basidiocarp of *H. erinaceus* possess antioxidant properties (Wong *et al*., 2009a). It is probably due to the antioxidant property of *H. erinaceus* that could be linked to its gastroprotective effect. These findings reveal that *H. erinaceus* aqueous extract could have components that prevented the formation of ulcer or reduced the severity of ulcer induced by ethanol. Because of their antioxidant properties as shown in the present study, their capacity to protect the mucosal tissue and their ability to prevent the depletion of antioxidant enzymes it is clear that *H. erinaceus* aqueous extract have gastroprotective activity.

4.3 Ulcer healing activity of *H. erinaceus* extracts in rats with ethanol - induced ulcers

4.3.1 Healing effect of *H. erinaceus* in gross gastric lesions evaluation

Ulcer treatment with *H. erinaceus* extract for three days using ethanol-induced gastric lesion model is shown in Plate 4.6. Results showed that rats oral-administered with omeprazole or *H. erinaceus* extracts had significantly reduced areas of gastric ulcer formation compared with ulcer control group (Plate 4.6b). Both *H. erinaceus* extracts significantly healed the lesions induced by ethanol. It was also observed that healing of gastric mucosa was the most prominent in rats treated with 400 mg/kg mushroom extract (Plate 4.6e).

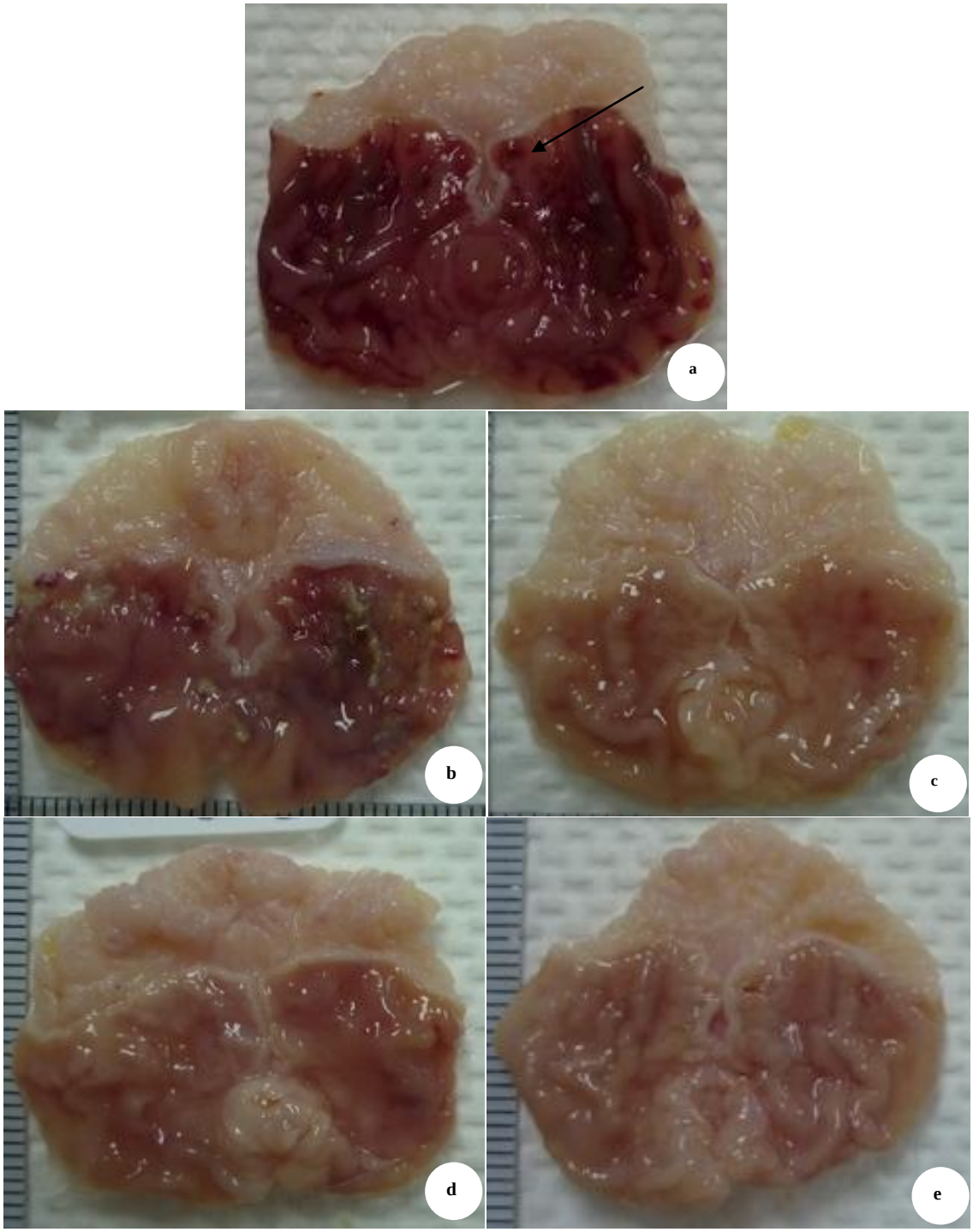

Plate 4.6: Gross appearance of the gastric mucosa in rats. (a) Rats treated with 5ml/kg distilled water (normal control group). Severe injuries are observed in the gastric mucosa. Ethanol produced extensive visible hemorrhagic necrosis (arrow) of gastric mucosa. (b) Rats treated with 5ml/kg distilled water (ulcer control group). Severe injuries are observed in the gastric mucosa. No healing activity is seen in this group. (c) Rats treated with omeprazole (20mg/kg). Injuries to the gastric mucosa are milder compared to the injuries seen in the ulcer control rats. (d). Rats treated with *H. erinaceus* extract (200 mg/kg). Very mild injuries are seen in the gastric mucosa. The extract heals the wound on gastric lesions induced by ethanol. (e). Rats treated with *H. erinaceus* extract (400 mg/kg). No injuries are seen in the gastric mucosa. The extract heals the wound on gastric lesions induced by ethanol.

4.3.2 Healing effect of *H. erinaceus* in the ulcer area in gastric glandular tissue

The healing effect was assessed by animal model. The rats were divided into five groups, Group 1 (ethanol only), Group 2 (ethanol + distilled water), Group 3 (ethanol + Omeprazole), Group 4 (ethanol + *H. erinaceus* extract 200mg/kg) and Group 5 (ethanol + *H. erinaceus* extract 400mg/kg). The results of a three days treatment of gastric ulcer induced by 95% ethanol in rats are shown in Figure 4.10. *Hericium erinaceus* extract treated groups had a significantly reduced ($p < 0.05$) ulcer area than that of distilled water and omeprazole groups. These results showed that in *H. erinaceus* extract treated groups at a dose of 400mg/kg the ulcer was healed as no ulcer was found on the gastric mucosa. Group 1 animals sacrificed at day zero had the largest ulcer area, that is 1436.4 mm^2. Group 4 (extract treated) animals showed mild inflammatory infiltration in the lamina propria, where as in the group 5 animals no ulcer area was found. During three days administration of *H. erinaceus* extract, ulcer area in rats were decreased in a dose dependent manner. These results showed strong healing effects of *H. erinaceus* extract which were observed in both the macroscopic and morphological analyses of the injuries. Similar result was shown by Mohamad Omar *et al.* (2011). *Lentinus squarrosulus* completely healed ethanol-induced ulcer rats during three days treatment, and found that the mushroom exhibited strong inhibitory action on serum IL-1β levels. This cytokine is involved in the induction of inflammation in gastric ulcer and as a main factor to induce recurrence of ulcer (Watanabe *et al.*, 2001). It was suggested that bioactive components of *H. erinaceus* extract may inhibit the production of IL-1β and accelerated the rate of ulcer healing in rats. However, this was not investigated in this study. Granulation tissue develops at the ulcer base two or three days after ulceration [(Tarnawski *et al*., 1990; Tarnawski *et al*., 1991; Tarnawski, (1993,

2000); and Cotran *et al*., 1999)]. This may explain rats treated with distilled water was significantly lower ($p < 0.05$) on day three as compared to day one.

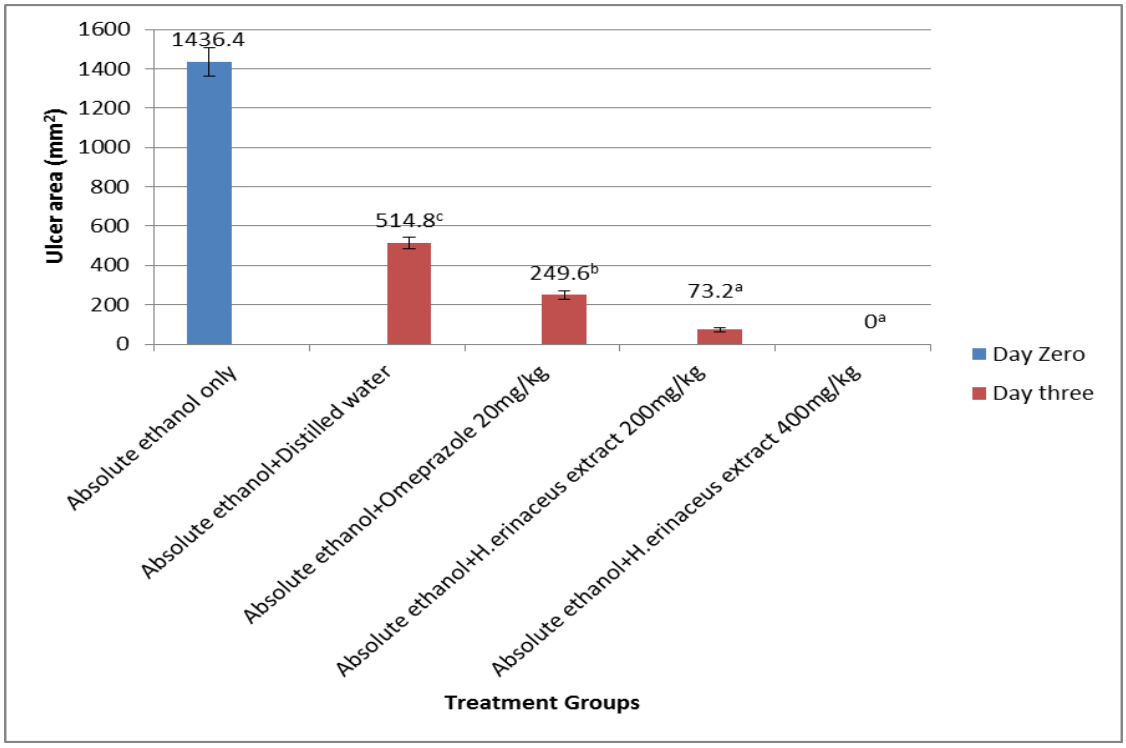

Figure 4.10: Effect of *H. erinaceus* extract on ulcer area in ethanol induced rats after three days treatment. All dosages were oral- administered in mg/kg daily. All values were expressed as mean of six replicate values. Mean with letters were significantly different ($p < 0.05$) by tukey Test. Six replicates animals were in each treatment group.

4.3.3 Effect of *H. erinaceus* on mucus production in gastric glandular tissue

Figure 4.11 demonstrated that the effect of *Hericium* extracts and omeprazole on mucus production at three days treatment after 95% ethanol induced gastric lesion. Rats that were treated with omeprazole produced the highest amount of mucus, that is 0.48g and followed by group treated with *Hericium* extract at a dose of 400mg/kg, 0.28g of mucus. The gastric mucosa contains many deep as well as surface glands. Chief and parietal cells are located in the body and fundus of stomach, while glands in the pyloric regions the standard drug and mushroom extract enhance the mucus secretion during the

imbalance. However, both treatment (group 4 and 5) groups did not show significant difference ($p < 0.05$) in mucus production after three days treatment.

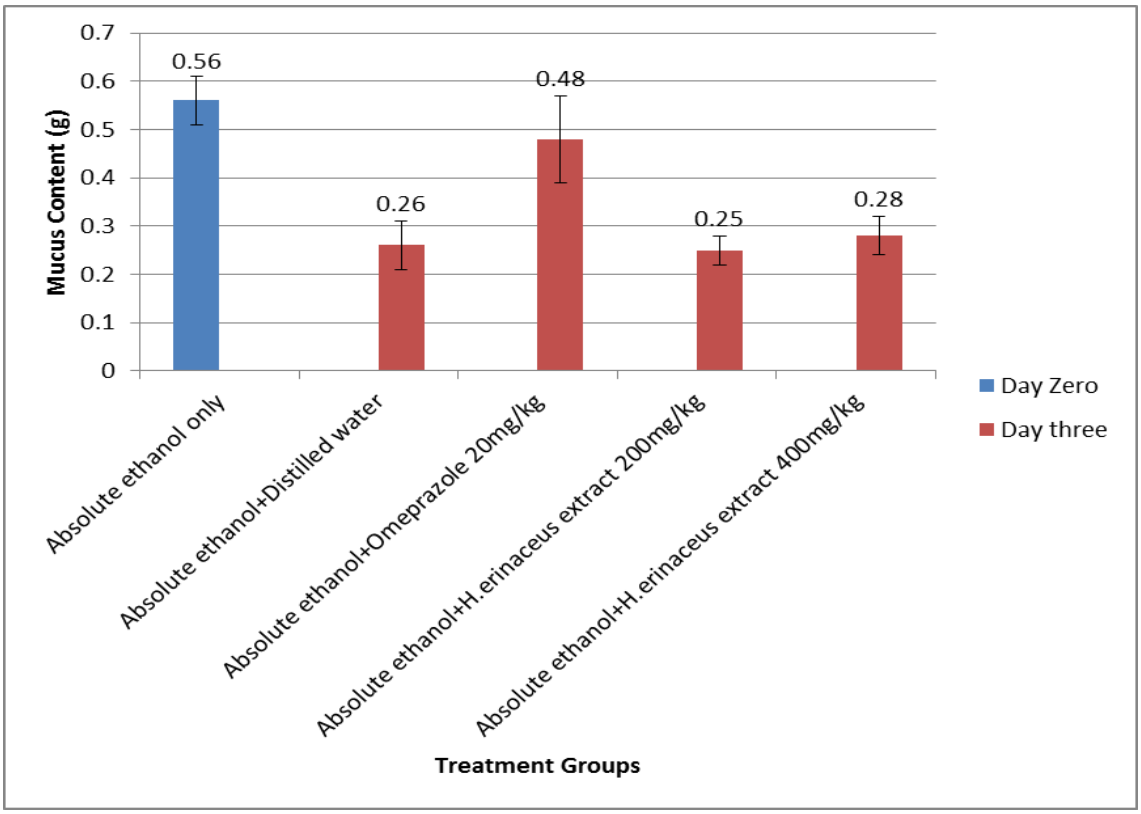

Figure 4.11: Effect of *H. erinaceus* extract on mucus production in ethanol induced rats after three days treatment. All dosages were oral- administered in mg/kg daily. All values were expressed as mean of six replicate values. Mean with letters were significantly different ($p < 0.05$) by Tukey Test. Six replicates animals were in each treatment group.

Koh and Chen (2000) have suggested that the proton pump inhibitor such as omeprazole could inhibit acid secretion and continuously induce production in plasma gastrin level thus involved in the regulation of mucosal cell proliferation during ulcer healing. Schmassmann and Reubi (2000) demonstrated that during ulcer healing process, omeprazole induced hypergastrinaemia, and exogenous gastrin-17 enhanced cell

proliferation in the ulcer margin. From this result, *H. erinaceus* extract may also possess similar activity with omeprazole, which secrete mucus during ulcer healing process.

4.3.4 Effect of *H. erinaceus* on gastric barrier mucus production in gastric glandular tissue of rats with ethanol-induced ulcer

The alcian blue binding capacity in *Hericium* extracts and omeprazole is shown in Figure 4.12. As shown in Figure 4.12, *Hericium* extract at a dose of 400mg/kg had the highest binding capacity of alcian blue, 8.29 μg/g, slightly higher than omeprazole group, which had 8.26 μg/g, then followed by 200mg/kg *Hericium* extract group, which recorded 7.58 μg/g. Rats treated with distilled water only had the lowest binding capacity of alcian blue after three days treatment. These results showed that *Hericium* extracts produced mucus which play an important role in gastric mucosa defense mechanism.

Laine *et al.* (2008) stated that mucus play a role in strengthening the mucosal barrier. During the healing process, mucus strength should increase or at least maintained to protect the regenerating gastric epithelium. Mucus layer protects newly formed cells against damage caused by acidic pH and proteolysis from gastric secretions (Kobayashi *et al.*, 2001; and Moraes & Hiruma-Lima, 2009). The ability of *H. erinaceus* extracts to induce mucus production promoted the accelerated healing of gastric mucosa in ethanol-induced rats.

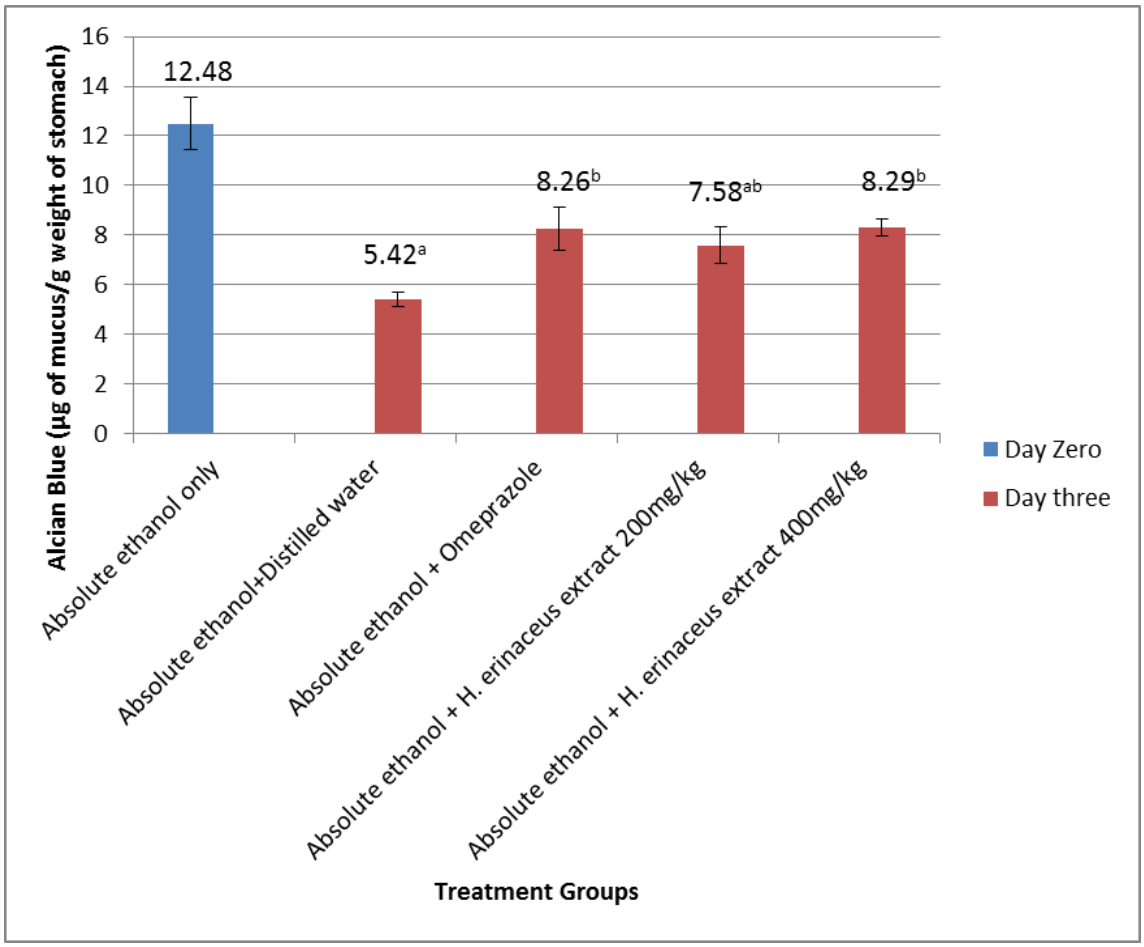

Figure 4.12: Effect of *H. erinaceus* extract on gastric barrier mucus in ethanol induced rats after three days treatment. All dosages were oral- administered in mg/kg daily. All values were expressed as mean of six replicate values. Mean with letters were significantly different ($p < 0.05$) by Tukey Test. Six replicates animals were in each treatment group.

4.3.5 Histological evaluation of gastric lesions in stomachs of control and treated rats

Histological observation of ethanol induced gastric lesions in ulcer control group treated with distilled water (normal control group) only showed severe damage to the epithelium and necrotic lesions penetrate deeply into mucosa, and extensive oedema and leucocytes infiltration of the submucosal layer were present (Plate 4.7). Rats that were treated with *H. erinaceus* extract had comparatively better healing effects of the gastric

mucosa as seen by reduction or absence of ulcer area, reduced or absence of submucosal edema and leucocytes infiltration (Plate 4.7).

Laine *et al.* (2008) stated that the reduction of neutrophils infiltration into ulcerated gastric tissues has been related in the promotion of healing of acetic-acid-induced chronic ulcer in rats. From the histological evaluation (Plate 4.7), it suggested that treatment with *H. erinaceus* extract promoted gastric ulcer healing when compared to the normal control group.

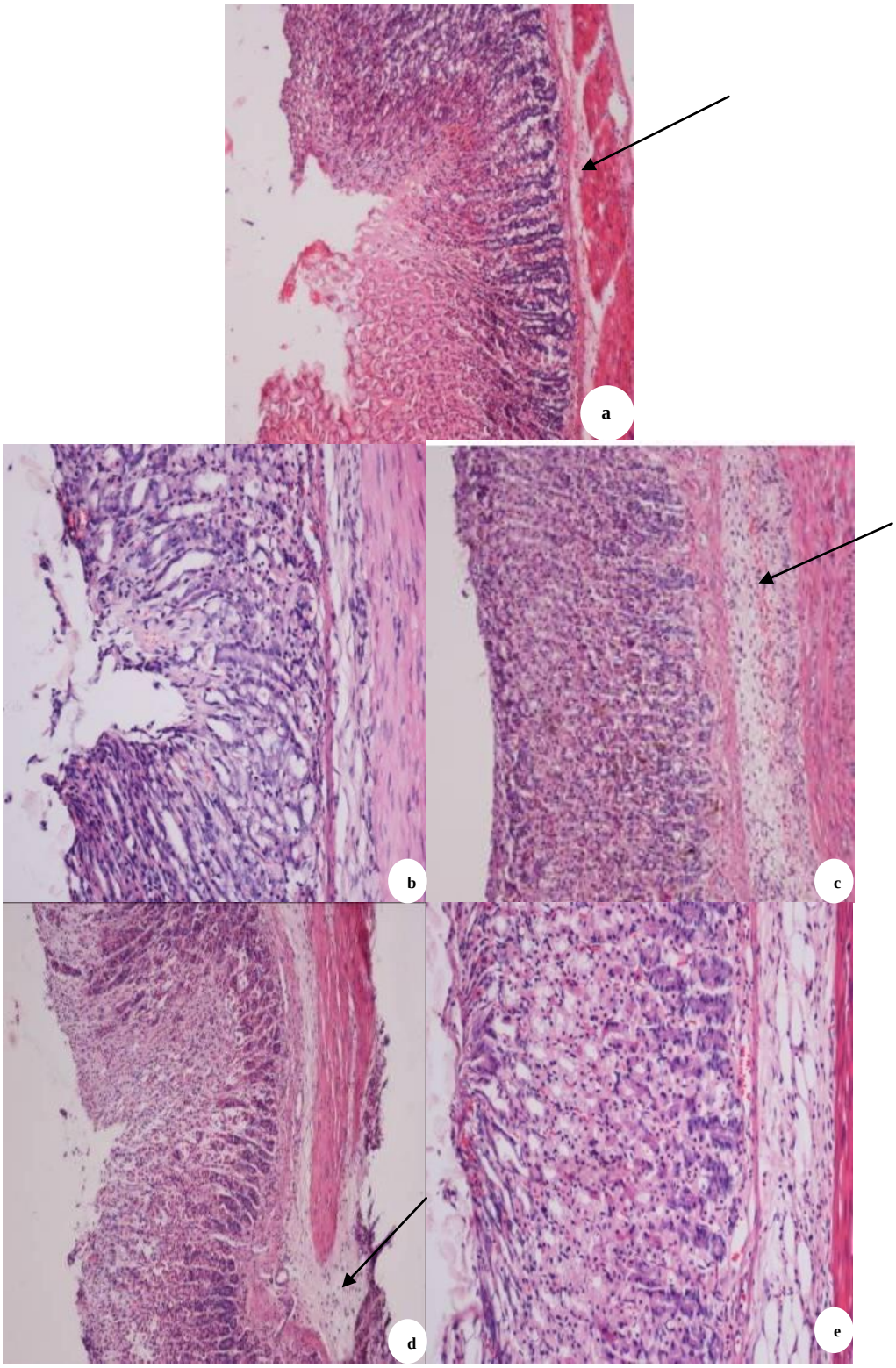

Plate 4.7: Histological study of ethanol-induced gastric mucosal damage in rats. (a) Rats treated with 5ml/kg distilled water (normal control group). There is severe disruption to the surface epithelium and necrotic lesions penetrating deeply into mucosa and extensive edema of submucosa layer and leucocytes infiltration is present. (b) Rats treated with 5ml/kg distilled water (ulcer control group). Moderate damage to the surface epithelium is seen. (c) Rats treated with omeprazole (20mg/kg). Mild disruption of the surface epithelium mucosa is seen. There is edema and leucocytes infiltration of the submucosal layer. (d) Rat treated with *H. erinaceus* extract (100 mg/kg). Mild disruption of surface epithelium is present. There is leucocytes infiltration. (e) Rats treated with *H. erinaceus* extract (400 mg/kg). There is no disruption to the surface epithelium and no edema or leucocytes infiltration of the submucosal layer (H&E stain 10× magnification). Notice the leucocytes infiltration in submucosa layer (arrows).

Mushrooms such as *Lentinula edodes*, *Pleurotus ostreatus* (Bilay *et al.*, 2011), *Ganoderma lucidum* (Gao *et al.*, 2002 and Rony *et al.*, 2011), *Lentinus squarrosulus* (Mohamad Omar *et al.*, 2011), *H. erinaceus* (Abdulla *et al.*, 2008) are reported to have gastroprotective property. However, only *G. lucidum* (Gao *et al.*, 2002) and *L. squarrosulus* (Mohamad Omar *et al.*, 2011) were shown to heal ethanol-induced gastric ulcer in rats.

In ulcer treatment, the two highest dosages of *H. erinaceus* extracts, that is 200mg/kg and 400mg/kg which were used in prevention test were selected. During the three days treatment, *H. erinaceus* extracts significantly ($p < 0.05$) reduced the ulcer area and healed the ulcers on gastric mucosa.

After ulceration is initiated, ulcer healing starts with a process of coagulation and hemostasis. The process is to prevent exsaguination and to provide a matrix for the cells coming into the ulcer in the later phase of healing (Chai, 2011). After two or three days, the process of granulation tissue formation takes place. Granulation tissue comprises of proliferation of connective tissue cells such as macrophage, fibroblast and proliferative endothelial cells which form microvessels through angiogenesis process (Tarnawski, 2005). Angiogenesis process is an important process in peptic ulcer healing and vascular endothelial growth factor (VEGF) is an important regulator that is involved in this process (Syam *et al.*, 2009).

Relationship between endothelial cells, platelets, coagulation, and fibrinolysis determines hemostasis and regulates the amount of fibrin deposited at the injury site, thus, the progress of healing were influenced by these factors. Growth factors, such as platelet derived growth factor (PDGF), transforming growth factor-β (TGF-β), epidermal growth factor (EGF), insulin-like growth factor (IGF), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), Tumor necrosis

factor-α (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6) are major stimulators for cells to go through proliferation, mitosis, migration, and re-epithelization (Chai, 2011). They act as promoters in the ulcer healing by activating and attracting neutrophils, macrophages, endothelial cells, fibroblasts and myofibroblasts to the ulcer area, and then bring the healing process to the inflammation phase. The inflammatory phase ends when lymphocytes are attracted to the ulcer by IL-1, an important regulator of collagenase activity that is later needed for extracellular matrix (ECM) remodelling.

ECM made of fibrous structural proteins such as the collagens and elastins, adhesive glycoproteins including fibronectin and laminin and an amorphous gel composed of proteoglycans and hyaluronan will form an interstitial matrix and the basement membrane (Basson *et al.*, 1993; Cotran *et al.*, 1999; and Basson, 2001). Later phase, ECM proteins will be degraded and replaced by matrix metalloproteinases (MMPs). Activated MMPs are inhibited by their antagonists, tissue inhibitors of metalloproteinase (TIMP), which can be found in most mesenchymal cells, thus preventing uncontrolled action of these proteinases (Cotran *et al.*, 1999; and Calabro *et al.*, 2004). Both proteins are important in the remodelling of connective tissue necessary for the tissue defect repair and scar formation (Shanin *et al.*, 2001; and Calabro *et al.*, 2004).

5.0 Recommendations for further studies and conclusions

In the present study, the potential efficacy of the mushroom *H. erinaceus* to protect and / or heal the gastric ulcer in rats experimentally induced by ethanol was investigated and compared with. Other models of gastric ulcer induction such as Hydrochloric acid (HCl), acetic acid or pylorus ligation methods can be conducted in the future. Other than that, biomarkers such as NF-kB, Interleukin-1β, TNF-α, PGE_2, NO and COX-2 can also be investigated to aid in understanding of the healing mechanism/s. Another staining method, Periodic acid-Schiff (PAS) staining can be alternative choice to evaluate the mucus secretion, which form a mucus layer to protect gastric mucosa against physical damage.

Furthermore, double-boiled aqueous extract was used in this study. Aqueous extract can be further purified to obtain pure compounds so that specific compounds which have effect on gastric ulcer treatment can be identified. Since aqueous extraction mainly release primary active compounds such as polysaccharides or beta glucans, solvents such as methanol and ethanol can be used to extract *H. erinaceus* basidiocarp to obtain secondary metabolites. Through alcohol extraction, secondary metabolite such as triterpenoids, which are believed to have anti-inflammatory property, may be obtained.

In conclusion, *Hericium erinaceus* did not show any sign of toxicological effects on rats during 14 days at a dose of 2 and 5 g/kg. *Hericium erinaceus* extract had gastroprotective activity in a dose dependent manner and produced mucus which is important in gastric defense mechanism. At 400mg/kg, there was a significant ($p < 0.05$) reduction of ulcer areas in the gastric wall as well as by the reduction or inhibition of edema and leukocytes infiltration of the submucosal layers which were shown histologically. Immunohistochemistry staining of HSP70 and Bax proteins showed up-

regulation of HSP70 protein and down-regulation of Bax protein in rats pre-treated with mushroom extract, in a dose dependent manner.

Beside that, *H. erinaceus* extract also significantly increased the SOD and CAT and decreased the level of lipid peroxidation (LPO) in gastric tissue homogenates. Among the extract concentrations, highest SOD and CAT enzyme activity were shown at 200mg/kg dose. In addition, *H. erinaceus* extract at 400mg/kg had the lowest LPO activity, and this result was comparable to omeprazole group.

During the three days ulcer treatment experiment, *H. erinaceus* extract at a dose of 400mg/kg could significantly ($p < 0.05$) heal the ulcer that was induced by ethanol in rats. No ulcer was observed after three days treatment and significantly higher ($p < 0.05$) mucus content was present when compared to extract at 200mg/kg dosage.

Hericium erinaceus, a well-documented temperate mushroom that is beneficial for gastrointestinal health in Chinese medicine is now grown in Malaysia. The gastroprotection and ulcer healing effects are retained though mushroom is grown at much higher temperatures in tropical Malaysia. All these results suggest the safety and efficacy of *H. erinaceus* either in preventing or healing gastric ulcer.

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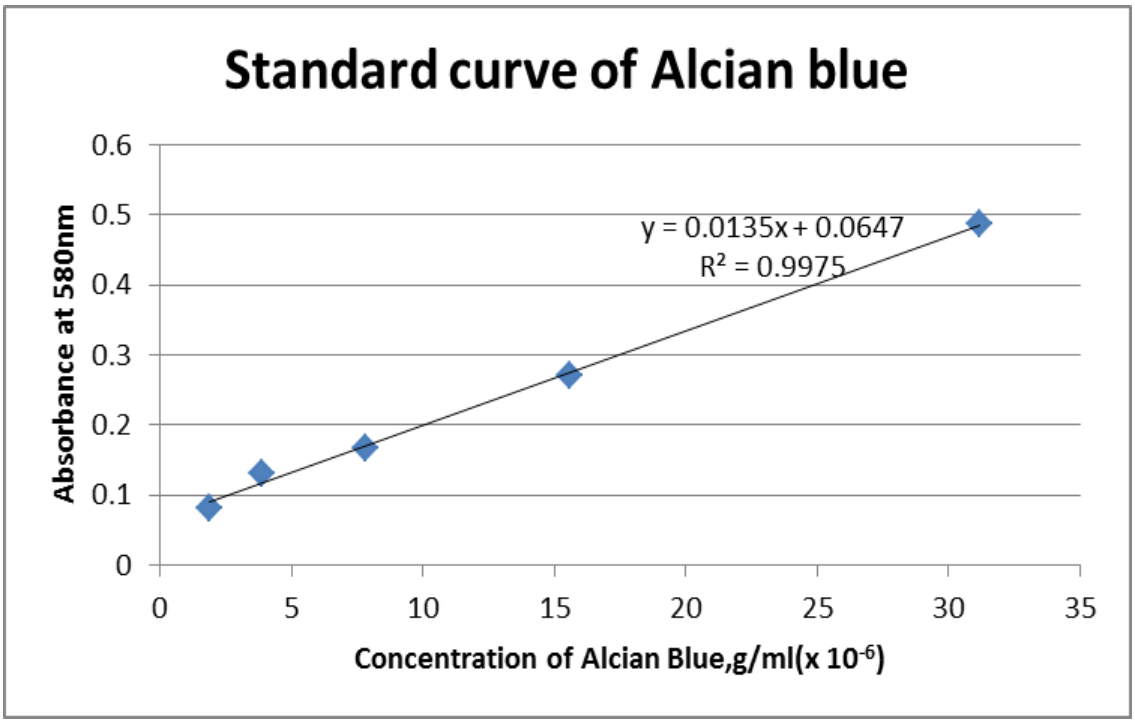
Appendix A: Analytical Methods

a) Gastric mucus barrier

Dissolve 1g of alcian blue in 100ml dH_2O, do a series of two times dilution. Rats stomach tissue homogenated in PBS buffer at a 1:8 ratio, that is 1g of tissue homogenated in 8 ml of PBS buffer.

Concentration (g/ml, $x10^{-6}$)	Absorbance at 580nm
1.9	0.0825
3.9	0.1305
7.8	0.167
15.6	0.271
31.2	0.487


Standard curve of Alcian blue
Absorbance at 580nm
0.6
0.5
0.4
0.3
0.2
0.1
0
y = 0.0135x + 0.0647
R² = 0.9975
0
5
10
15
20
25
30
35
Concentration of Alcian Blue,g/ml(x 10-6)

b) Bradford Test

Prepare stock standard 1mg/ml (5mg/5ml) of BSA

Prepare three to five times dilution

1mg/ml

↓

0.5mg/ml

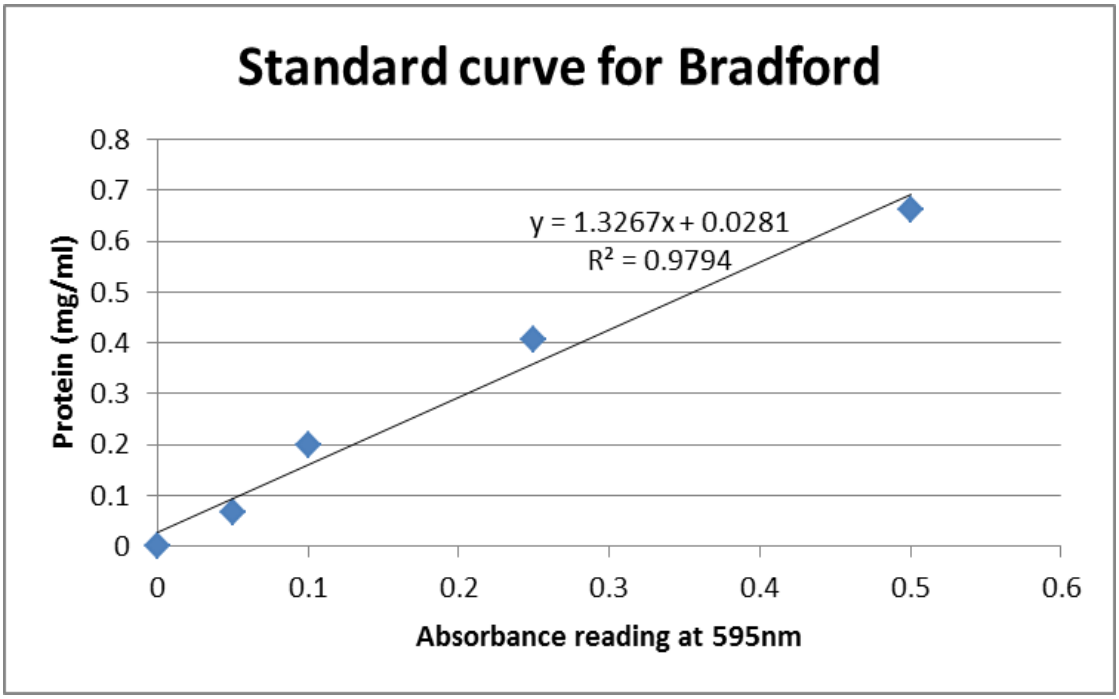
↓

0.1mg/ml

↓

0.05mg/ml

Concentration of protein (mg/ml)	Absorbance at 595nm
0	0
0.05	0.06633
0.1	0.197667
0.25	0.407667
0.5	0.663



Standard curve of Bradford was plotted after five times dilution of tissue homogenated solution.

c) Lipid peroxidation assay: Standard preparation

12.5 µl TEP/ 50ml dH_2O

↓

20 µM

2x dilution ↙ ↘

10µM 15 µM

2x dilution ↓

5 µM

2x dilution ↓

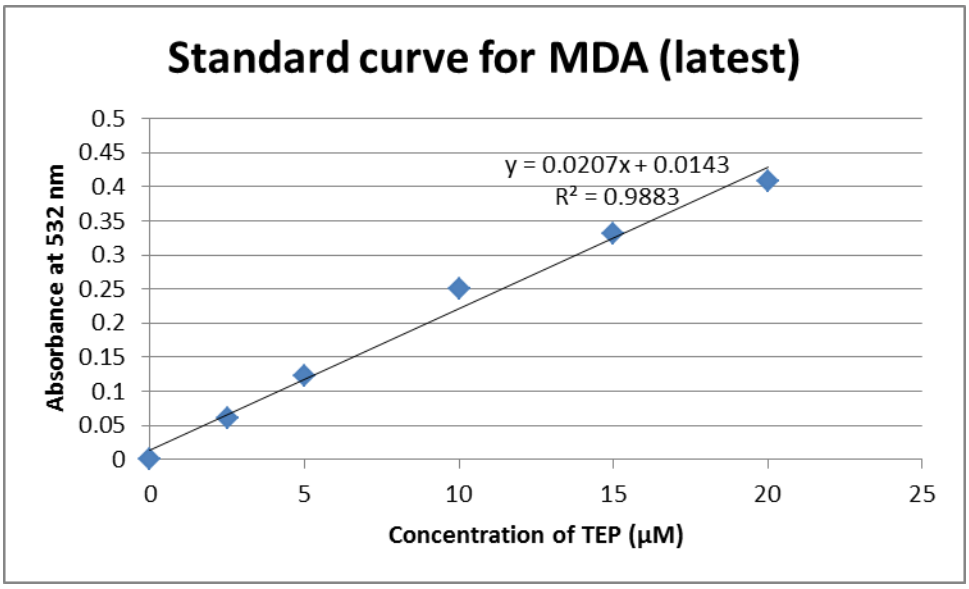
2.5 µM

Reagent preparation

15% TCA : Dissolve 6g of TCA in 40ml dH_2O

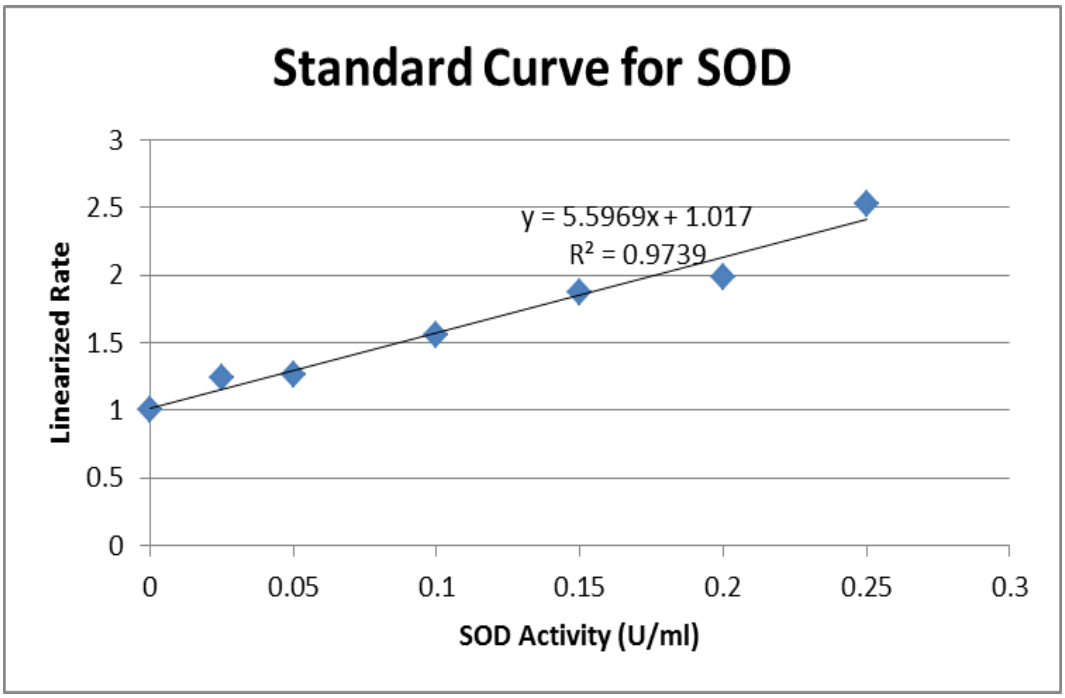
1% TBA : Dissolve 0.4g of TBA in 40ml dH_2O

Concentration of TEP (µm)	Absorbance at 532 nm
0	0
2.5	0.06
5	0.123
10	0.25
15	0.332
20	0.409



d) SOD

SOD activity (U/ml)	Linearized rate
0	1
0.025	1.243644
0.05	1.262366
0.1	1.56117
0.15	1.869427
0.2	1.989831
0.25	2.530172

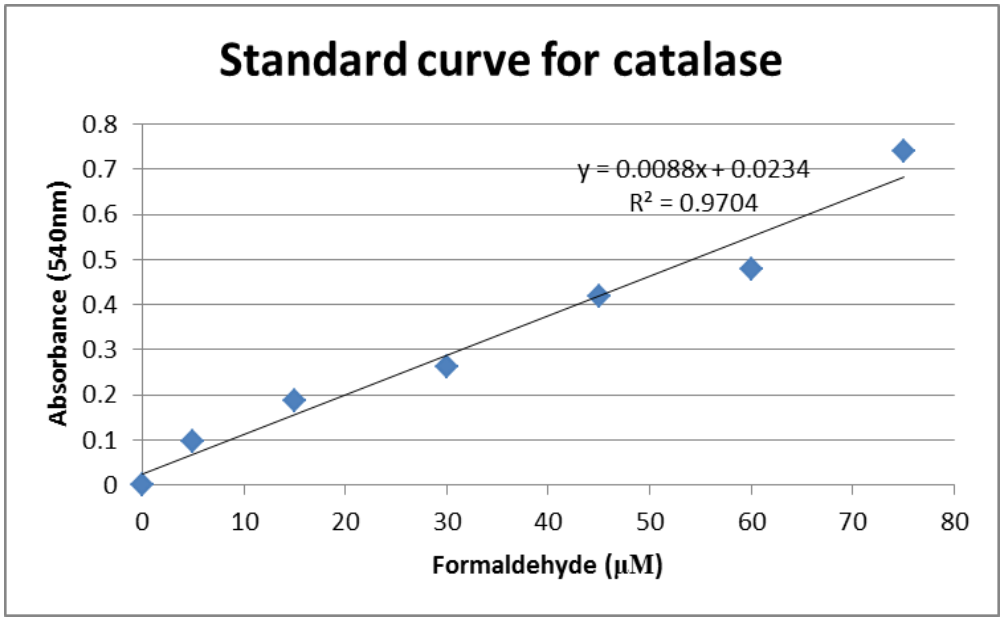


Standard curve of SOD

$$\text{SOD (U/ml)} = \left[\left(\frac{\text{Sample LR} - \text{y-intercept}}{\text{slope}} \right) X \frac{0.23 \text{ ml}}{0.01 \text{ ml}} \right] X \text{ Sample dilution}$$

e) CAT

Formaldehyde (µM)	Absorbance at 540 nm
0	0
5	0.096
15	0.1875
30	0.262
45	0.4185
60	0.48
75	0.7405



$$\text{Formaldehyde } (\mu M) = \left[\frac{\text{Sample absorbance} - (\text{y-intercept})}{\text{slope}} \right] X \frac{0.17 \text{ ml}}{0.02 \text{ ml}}$$

$$\text{CAT Activity} = \frac{\mu M \text{ of sample}}{20 \text{ min.}} X \quad \text{Sample dilution} = \text{nmol/min/ml}$$

f) Histological technique

1. Specimen preparation

- Trim tissue into small size.
- Insert the trimming tissue into cassette and label properly.
- Place the cassettes in the 10% buffered formalin.

2. Automated tissue processing

Process	Duration (hours)
Dehydration	
1. 10% Formalin	1
2. 10% Formalin	1
3. 70% Ethanol 1	1
4. 95% Ethanol 2	1
5. 95% Ethanol 3	1
6. 95% Ethanol 4	1
7. Absolute Ethanol 1	1
8. Absolute Ethanol 2	$1_{1/2}$
CLEARING	
9. Alcohol :Xylene (1:1)	1
10. Xylene 1	$1_{1/2}$
11. Xylene 2	$1_{1/2}$
EMBEDDING	
12. Paraffin Wax 1	$1_{1/2}$
13. Paraffin Wax 1	$1_{1/2}$
TOTAL	$14_{1/2}$

3. Tissue blocking

- Place the tissue in mold properly
- Load the paraffin wax into the mold.
- Place the mold on cold plate area and wait until it solidifies.

4. Sectioning

- Section the tissue blocking by using a rotary microtome to a thickness of 5 µm.
- Put the ribbon section onto 42 °C water bath.
- Pick the best tissue section on water bath by using clean glass slide.
- Dehydrate the slides in an oven at 55°C for at least 24 hours.

5. Staining

a) Haematoxylin and Eosin staining

Process (solution)	**Time duration (minutes)**
DEWAXING	
1. Xylene 1	3
2. Xylene 1	3
REHYDRATION	
3. Absolute Alcohol	2
4. 95%Alcohol 1	2
5. 95%Alcohol 2	2
6**.** 70%Alcohol	2

BRING SECTION TO WATER (RUNNING WATER)	3
STAINING 7. Harrls' Haematoxylin 8. Running Water 9. 0.5% Acid Alcohol 10. Running Water 11. 2% Sodium Acetate 12. Running Water 13. Rinse in 80% Alcohol 14. Eosin	 10 minutes Remove excess colour 2-3 dips 2-3 minutes 2 second 2-3 minutes 2-3 dips 5 minutes
DEHYDATION 15. 95%Alcohol 1 16. 95%Alcohol 2 17. Absolute Alcohol 1 18. Absolute Alcohol 2	 5 second 2 minutes 2 minutes 2 minutes
CLEARING 19. Xylene 1 20. Xylene 2 21. Xylene 3	 2 minutes 2 minutes 3 minutes

b) Immunohistochemistry staining

Process (solution)	**Time duration (minutes)**
DEWAXING 1. Xylene 1 2. Xylene 2 3. Xylene 3	 5 5 5
REHYDRATION 4. 95%Alcohol 5. 85%Alcohol 6. 70%Alcohol 7. Distilled water	 20 dips 20 dips 20 dips 5 minutes
WASHING 8. Soak in 10X TBS wash buffer and heat in oven at low temperature. 9. Cool down in room temperature 10. Tap off excess TBS buffer. 11. Use lintless tissue to wipe around the specimen.	 20
STAINING i) Peroxidase block • Tap off excess wash buffer. Using a lintless tissue to wipe around the specimen.	 5

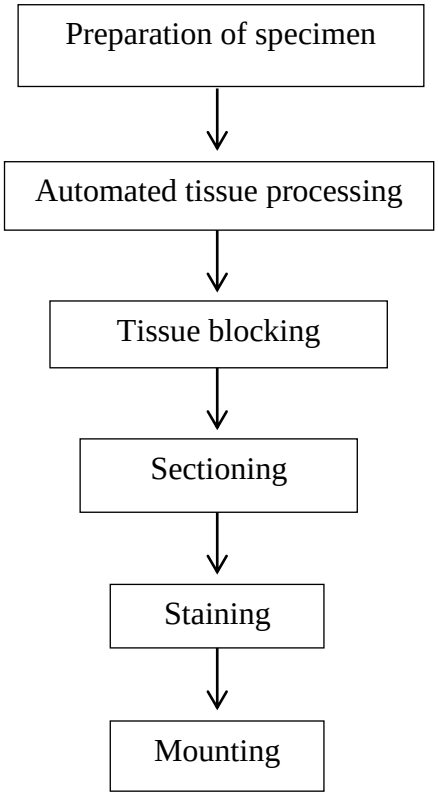
• Apply Peroxidase block to cover specimen • Incubate • Rinse gently with distilled water	
ii) Biotinylated primary antibody • Wipe slides as before. • Apply prepared biotinylated primary antibody to cover specimen • Incubate • Rinse gently with wash buffer and place in buffer bath	15 15
iii) Streptavidin peroxidase • Wipe slides as before. • Apply Streptavidin peroxidase to cover specimen • Incubate • Rinse gently with wash buffer and place in buffer bath	10 dips 2-5 minutes
iv) DAB+ substrate chromogen • Wipe slides as before • Apply prepared DAB+ substrate chromogen to cover specimen. • Incubate • Rinse gently with distilled water from wash bottle	

v) Haematoxylin counterstain • Immerse slides in a bath of haematoxylin. • 0.037mol/L ammonia • Rinse slides in a bath of distilled water	

6. Mounting

Mount stained tissue with DPX mounting media and cover the tissue with cover slip and wipe the slide to remove excess xylene and then observed under light microscope**.**

Flow chart of histological technique



Appendix B: Results of Experiments

a) Acute toxicity test

i) Body weight of rat

Table 1. Descriptive: body weight of male rats in control group (Treatment)

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Day 0	6	247.5000	33.88067	13.83173	211.9444	283.0556	190.00	280.00
Day 7	6	278.6667	29.83063	12.17830	247.3613	309.9720	230.00	305.00
Day 14	6	288.6667	28.40892	11.59789	258.8533	318.4800	241.00	313.00
Total	18	271.6111	34.09009	8.03511	254.6585	288.5637	190.00	313.00

Table 2. ANOVA: body weight of male rats in control group (Treatment)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5532.111	2	2766.056	2.917	.085
Within Groups	14224.167	15	948.278		
Total	19756.278	17			

Table 3. Multiple range tests: body weight of male rats in control group (Treatment)

	Group	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	Day 0	6	247.5000	
	Day 7	6	278.6667	
	Day 14	6	288.6667	
	Sig.		.084	

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

Table 4. Descriptive: body weight of male rats in low dose group

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Day 0	6	226.0000	13.84197	5.65096	211.4737	240.5263	210.00	242.00
Day 7	6	247.6667	15.40995	6.29109	231.4949	263.8384	225.00	263.00
Day 14	6	250.1667	19.23972	7.85458	229.9758	270.3575	225.00	270.00
Total	18	241.2778	18.96686	4.47053	231.8458	250.7098	210.00	270.00

Table 5. ANOVA: body weight of male rats in low dose group

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2119.444	2	1059.722	3.978	.041
Within Groups	3996.167	15	266.411		
Total	6115.611	17			

Table 6. Multiple range tests: body weight of male rats in low dose group

Tukey HSD[a]	Group	N	Subset for alpha = 0.05	
			1	2
	Day 0	6	226.0000	
	Day 7	6	247.6667	
	Day 14	6	250.1667	
	Sig.		.053	

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

Table 7. Descriptive: body weight of male rats in high dose group

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Day 0	6	229.3333	38.89302	15.87801	188.5176	270.1491	176.00	280.00
Day 7	6	262.5000	46.51344	18.98903	213.6871	311.3129	203.00	319.00
Day 14	6	268.8333	49.32917	20.13855	217.0655	320.6011	204.00	325.00
Total	18	253.5556	45.98494	10.83875	230.6878	276.4233	176.00	325.00

Table 8. ANOVA: body weight of male rats in high dose group

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5400.778	2	2700.389	1.326	.295
Within Groups	30547.667	15	2036.511		
Total	35948.444	17			

Table 9. Multiple range tests: body weight of male rats in high dose group

	Group	N	Subset for alpha = 0.05
			1
Tukey HSD[a]	Day 0	6	229.3333
	Day 7	6	262.5000
	Day 14	6	268.8333
	Sig.		.312

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

Table 10. Descriptive: body weight of female rats in control group

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Day 0	6	186.3333	19.68417	8.03603	165.6761	206.9906	164.00	213.00
Day 7	6	189.6667	23.12286	9.43987	165.4007	213.9326	167.00	219.00
Day 14	6	195.5000	17.87456	7.29726	176.7418	214.2582	175.00	220.00
Total	18	190.5000	19.50339	4.59699	180.8012	200.1988	164.00	220.00

Table 11. ANOVA: body weight of female rats in control group

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	258.333	2	129.167	.312	.737
Within Groups	6208.167	15	413.878		
Total	6466.500	17			

Table 12. Multiple range tests: body weight of female rats in control group

	Group	N	Subset for alpha = 0.05
			1
Tukey HSD[a]	Day 0	6	186.3333
	Day 7	6	189.6667
	Day 14	6	195.5000
	Sig.		.720

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

Table 13. Descriptive: body weight of female rats in low dose group

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Day 0	6	216.3333	24.56556	10.02885	190.5534	242.1133	188.00	257.00
Day 7	6	218.1667	22.82469	9.31814	194.2136	242.1197	194.00	253.00
Day 14	6	224.0000	18.06654	7.37564	205.0403	242.9597	204.00	250.00
Total	18	219.5000	20.92915	4.93305	209.0922	229.9078	188.00	257.00

Table 14. ANOVA: body weight of female rats in low dose group

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	192.333	2	96.167	.199	.822
Within Groups	7254.167	15	483.611		
Total	7446.500	17			

Table 15. Multiple range tests: body weight of female rats in low dose group

	Group	N	Subset for alpha = 0.05
			1
Tukey HSD[a]	Day 0	6	216.3333
	Day 7	6	218.1667
	Day 14	6	224.0000
	Sig.		.820

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

Table 16. Descriptive: body weight of female rats in high dose group

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Day 0	6	199.8333	16.00521	6.53410	183.0369	216.6298	168.00	212.00
Day 7	6	202.6667	16.39105	6.69162	185.4653	219.8680	174.00	222.00
Day 14	6	207.3333	14.69240	5.99815	191.9146	222.7521	180.00	221.00
Total	18	203.2778	15.09891	3.55885	195.7693	210.7863	168.00	222.00

Table 17. ANOVA: body weight of female rats in high dose group

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	172.111	2	86.056	.349	.711
Within Groups	3703.500	15	246.900		
Total	3875.611	17			

Table 18. Multiple range tests: body weight of female rats in high dose group

	Group	N	Subset for alpha = 0.05
			1
Tukey HSD[a]	Day 0	6	199.8333
	Day 7	6	202.6667
	Day 14	6	207.3333
	Sig.		.693

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

ii) Biochemical tests

Parameters that showed significantly different only displayed as below:

Table 19. Descriptive: Renal function test of male rats in potassium (mmol/L)

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
G1	6	4.7667	.15055	.06146	4.6087	4.9247	4.60	5.00
G2	6	5.0833	.20412	.08333	4.8691	5.2975	4.90	5.30
G3	6	5.0833	.23166	.09458	4.8402	5.3264	4.80	5.50
Total	18	4.9778	.24146	.05691	4.8577	5.0979	4.60	5.50

Table 20. ANOVA: Renal function test of male rats in potassium (mmol/L)

	Sum of Squares	df.	Mean Square	F	Sig.
Between Groups	.401	2	.201	5.099	.020
Within Groups	.590	15	.039		
Total	.991	17			

Table 21. Multiple range tests: Renal function test of male rats in potassium (mmol/L)

	Group	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	G1	6	4.7667	
	G3	6		5.0833
	G2	6		5.0833
	Sig.		1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

Table 22. Descriptives: Liver function test of male rats in Alanine Aminotransferase (IU/L)

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
G1	6	51.8333	4.70815	1.92209	46.8924	56.7742	45.00	58.00
G2	6	48.0000	1.41421	.57735	46.5159	49.4841	46.00	50.00
G3	6	46.5000	3.56371	1.45488	42.7601	50.2399	42.00	50.00
Total	18	48.7778	4.02281	.94819	46.7773	50.7783	42.00	58.00

Table 23. ANOVA: Liver function test of male rats in Alanine Aminotransferase (IU/L)

	Sum of Squares	df.	Mean Square	F	Sig.
Between Groups	90.778	2	45.389	3.693	.050
Within Groups	184.333	15	12.289		
Total	275.111	17			

Table 24. Multiple range tests: Liver function test of male rats in Alanine Aminotransferase (IU/L)

	Group	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	G3	6	46.5000	
	G2	6	48.0000	48.0000
	G1	6		51.8333
	Sig.		.743	.175

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

Table 25. Descriptives: Liver function test of female rats in Alkaline Phosphatase (IU/L)

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
G1	6	114.8333	24.73392	10.09758	88.8767	140.7900	84.00	159.00
G2	6	72.8333	12.63988	5.16021	59.5686	86.0981	53.00	89.00
G3	6	94.0000	17.50429	7.14609	75.6304	112.3696	75.00	125.00
Total	18	93.8889	25.06671	5.90828	81.4235	106.3543	53.00	159.00

Table 26. ANOVA: Liver function test of female rats in Alkaline Phosphatase (IU/L)

	Sum of Squares	df.	Mean Square	F	Sig.
Between Groups	5292.111	2	2646.056	7.364	.006
Within Groups	5389.667	15	359.311		
Total	10681.778	17			

Table 27. Multiple range tests: Liver function test of female rats in Alkaline Phosphatase (IU/L)

	Group	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	G2	6	72.8333	
	G3	6	94.0000	94.0000
	G1	6		114.8333
	Sig.		.163	.172

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

b) Ulcer prevention:

i) Ulcer area

Table 28. Descriptives: ulcer area of rats in ulcer prevention test

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Positive Group	6	285.6000	48.11985	19.64485	235.1013	336.0987	223.20	360.00
Negative Group	6	894.0000	89.86069	36.68547	799.6970	988.3030	756.00	993.60
50mg/kg Group	6	727.2000	65.83179	26.87571	658.1138	796.2862	640.80	806.40
100mg/kg Group	6	602.4000	72.09594	29.43304	526.7400	678.0600	504.00	720.00
200mg/kg Group	6	453.6000	68.30520	27.88548	381.9181	525.2819	367.20	525.60
400mg/kg Group	6	240.0000	42.39245	17.30665	195.5118	284.4882	172.80	280.80
Total	36	533.8000	244.31641	40.71940	451.1352	616.4648	172.80	993.60

Table 29. ANOVA: ulcer area of rats in ulcer prevention test

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1957243.680	5	391448.736	89.017	.000
Within Groups	131924.160	30	4397.472		
Total	2089167.840	35			

Table 30. Multiple range tests: ulcer area of rats in ulcer prevention test

	Group	N	Subset for alpha = 0.05				
			1	2	3	4	5
Tukey HSD[a]	400mg/kg Group	6	240.0000				
	Positive Group	6	285.6000				
	200mg/kg Group	6		453.6000			
	100mg/kg Group	6			602.4000		
	50mg/kg Group	6				727.2000	
	Negative Group	6					894.0000
	Sig.		.838	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

ii) Mucus content

Table 31. Descriptives: mucus content of rats in ulcer prevention test

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Normal Group	6	.4271	.14234	.05811	.2777	.5765	.21	.59
Positive Group	6	1.5557	.16984	.06934	1.3774	1.7339	1.37	1.85
Negative Group	6	.7988	.07577	.03093	.7193	.8783	.70	.89
50mg/kg Group	6	.7961	.07279	.02971	.7197	.8725	.70	.90
100mg/kg Group	6	.8207	.09700	.03960	.7189	.9225	.64	.93
200mg/kg Group	6	.9266	.15024	.06133	.7689	1.0842	.76	1.15
400mg/kg Group	6	1.1833	.09702	.03961	1.0815	1.2851	1.04	1.32
Total	42	.9297	.35066	.05411	.8205	1.0390	.21	1.85

Table 32. ANOVA: mucus content of rats in ulcer prevention test

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4.534	6	.756	52.093	.000
Within Groups	.508	35	.015		
Total	5.041	41			

Table 33. Multiple range tests: mucus content of rats in ulcer prevention test

	Group	N	Subset for alpha = 0.05			
			1	2	3	4
Tukey HSD[a]	Normal Group	6	.4271			
	50mg/kg Group	6		.7961		
	Negative Group	6		.7988		
	100mg/kg Group	6		.8207		
	200mg/kg Group	6		.9266		
	400mg/kg Group	6			1.1833	
	Positive Group	6				1.5557
	Sig.		1.000	.509	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.00

iii) Alcian blue

Table 34. Descriptives: alcian blue of rats in ulcer prevention test

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Non Treatment Group	6	5.0099	1.12988	.46127	3.8241	6.1956	3.21	6.10
Positive Group	6	6.7259	1.64754	.67261	4.9969	8.4549	4.91	9.13
Negative Group	6	3.4173	.31572	.12889	3.0860	3.7486	2.84	3.80
Extract 50mg/kg Group	6	8.0963	1.70917	.69777	6.3026	9.8900	6.17	10.24
Extract 100mg/kg Group	6	11.1951	1.09611	.44749	10.0448	12.3454	9.87	12.76
Extract 200mg/kg Group	6	12.2938	1.49231	.60923	10.7277	13.8599	10.02	14.02
Extract 400mg/kg Group	6	10.6395	2.21265	.90331	8.3175	12.9615	8.39	13.58
Total	42	8.1968	3.41509	.52696	7.1326	9.2610	2.84	14.02

Table 35. ANOVA: alcian blue of rats in ulcer prevention test

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	401.495	6	66.916	30.543	.000
Within Groups	76.681	35	2.191		
Total	478.176	41			

Table 36. Multiple range tests: alcian blue of rats in ulcer prevention test

	Group	N	Subset for alpha = 0.05				
			1	2	3	4	5
Tukey HSD[a]	Negative Group	6	3.4173				
	Non Treatment Group	6	5.0099	5.0099			
	Positive Group	6		6.7259	6.7259		
	Extract 50mg/kg Group	6			8.0963	8.0963	
	Extract 400mg/kg Group	6				10.6395	10.6395
	Extract 100mg/kg Group	6					11.1951
	Extract 200mg/kg Group	6					12.2938
	Sig.		.517	.428	.681	.071	.472

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

iv) LPO

Table 37. Descriptives: LPO of rats in ulcer prevention test

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Non Treatment Group	3	4.4300	.12781	.07379	4.1124	4.7475	4.29	4.53
Positive Group	3	5.4444	.17418	.10056	5.0118	5.8771	5.30	5.64
Negative Group	3	8.8905	.16966	.09795	8.4691	9.3119	8.73	9.07
Extract 50mg/kg Group	3	7.4573	.26607	.15361	6.7964	8.1183	7.18	7.71
Extract 100mg/kg Group	3	6.8293	.24790	.14313	6.2135	7.4451	6.56	7.04
Extract 200mg/kg Group	3	6.0242	.25563	.14759	5.3891	6.6592	5.73	6.22
Extract 400mg/kg Group	3	5.2512	.12781	.07379	4.9337	5.5687	5.11	5.35
Total	21	6.3324	1.44504	.31533	5.6746	6.9902	4.29	9.07

Table 38. ANOVA: LPO of rats in ulcer prevention test

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	41.184	6	6.864	166.033	.000
Within Groups	.579	14	.041		
Total	41.763	20			

Table 39. Multiple range tests: LPO of rats in ulcer prevention test

			Subset for alpha = 0.05					
	Group	**N**	1	2	3	4	5	6
Tukey HSD[a]	Non Treatment Group	3	4.4300					
	Extract 400mg/kg Group	3		5.2512				
	Positive Group	3		5.4444				
	Extract 200mg/kg Group	3			6.0242			
	Extract 100mg/kg Group	3				6.8293		
	Extract 50mg/kg Group	3					7.4573	
	Negative Group	3						8.8905
	Sig.		1.000	.896	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

v) SOD

Table 40. Descriptives: SOD of rats in ulcer prevention test

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Non Treatment Group	3	8.8161	.53526	.30903	7.4865	10.1458	8.26	9.33
Positive Group	3	5.7701	.34725	.20049	4.9074	6.6327	5.37	5.98
Negative Group	3	1.8890	.28329	.16356	1.1852	2.5927	1.64	2.20
Extract 50mg/kg Group	3	2.1032	.25757	.14871	1.4633	2.7430	1.90	2.39
Extract 100mg/kg Group	3	4.3537	.58925	.34021	2.8899	5.8175	3.95	5.03
Extract 200mg/kg Group	3	6.2390	.66930	.38642	4.5764	7.9016	5.54	6.87
Extract 400mg/kg Group	3	5.3168	.47054	.27167	4.1480	6.4857	4.79	5.69
Total	21	4.9268	2.33308	.50912	3.8648	5.9888	1.64	9.33

Table 41. ANOVA: SOD of rats in ulcer prevention test

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	105.725	6	17.621	78.550	.000
Within Groups	3.141	14	.224		
Total	108.866	20			

Table 42. Multiple range tests: SOD of rats in ulcer prevention test

			Subset for alpha = 0.05				
	Group	**N**	1	2	3	4	5
Tukey HSD[a]	Negative Group	3	1.8890				
	Extract 50mg/kg Group	3	2.1032				
	Extract 100mg/kg Group	3		4.3537			
	Extract 400mg/kg Group	3		5.3168	5.3168		
	Positive Group	3			5.7701		
	Extract 200mg/kg Group	3			6.2390		
	Non Treatment Group	3				8.8161	
	Sig.		.997	.234	.273	1.000	

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

vi) CAT

Table 43. Descriptives: CAT of rats in ulcer prevention test

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Non treatment Group	3	179.2439	20.59684	11.89159	128.0785	230.4093	166.61	203.01
Positive Group	3	68.5147	6.52168	3.76529	52.3139	84.7154	62.32	75.32
Negative Group	3	112.1032	15.27800	8.82076	74.1505	150.0558	100.08	129.30
Extract 50mg/kg Group	3	40.4522	4.68744	2.70629	28.8080	52.0965	37.60	45.86
Extract 100mg/kg Group	3	96.2136	18.41117	10.62970	50.4778	141.9495	83.11	117.26
Extract 200mg/kg Group	3	135.9413	11.31900	6.53503	107.8233	164.0592	123.84	146.27
Extract 400mg/kg Group	3	126.0888	10.11856	5.84195	100.9529	151.2246	114.56	133.49
Total	21	108.3654	44.70120	9.75460	88.0176	128.7131	37.60	203.01

Table 44. ANOVA: CAT of rats in ulcer prevention test

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	37380.684	6	6230.114	33.764	.000
Within Groups	2583.256	14	184.518		
Total	39963.940	20			

Table 45. Multiple range tests: CAT of rats in ulcer prevention test

			Subset for alpha = 0.05				
	Group	**N**	1	2	3	4	5
Tukey HSD[a]	Extract 50mg/kg Group	3	40.4522				
	Positive Group	3	68.5147	68.5147			
	Extract 100mg/kg Group	3		96.2136	96.2136		
	Negative Group	3			112.1032	112.1032	
	Extract 400mg/kg Group	3			126.0888	126.0888	
	Extract 200mg/kg Group	3				135.9413	
	Non treatment Group	3					179.2439
	Sig.		.220	.231	.171	.378	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

c) Ulcer healing

i) Ulcer area

Table 46. Descriptives: ulcer area of rats in ulcer treatment test

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Ulcer Control Group	6	514.8000	70.36135	28.72490	440.9603	588.6397	403.20	590.40
Omeprazole Group	6	249.6000	56.07994	22.89454	190.7477	308.4523	158.40	324.00
Hericium extract 200mg/kg	6	73.2000	22.95735	9.37230	49.1077	97.2923	43.20	108.00
Hericium extract 400mg/kg	6	.0000	.00000	.00000	.0000	.0000	.00	.00
Total	24	209.4000	207.13549	42.28136	121.9344	296.8656	.00	590.40

Table 47. ANOVA: ulcer area of rats in ulcer treatment test

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	943704.000	3	314568.000	145.925	.000
Within Groups	43113.600	20	2155.680		
Total	986817.600	23			

Table 48. Multiple range test: ulcer area of rats in ulcer treatment test

	Group	N	Subset for alpha = 0.05			
			1	2	3	4
Tukey HSD[a]	Hericium extract 400mg/kg	6	.0000			
	Hericium extract 200mg/kg	6	73.2000			
	Omeprazole Group	6		249.6000		
	Ulcer Control Group	6			514.8000	
	Sig.		.057	1.000	1.000	

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

ii) Mucus content

Table 49. Descriptives: mucus content of rats in ulcer treatment test

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Ulcer Control Group	6	.2600	.11462	.04679	.1398	.3803	.11	.40
Omeprazole Group	6	.4813	.23871	.09745	.2308	.7318	.25	.92
Hericium extract 200mg/kg	6	.2458	.08314	.03394	.1585	.3330	.10	.34
Hericium extract 400mg/kg	6	.2790	.09066	.03701	.1839	.3742	.13	.38
Total	24	.3165	.16770	.03423	.2457	.3873	.10	.92

Table 50. ANOVA: mucus content of rats in ulcer treatment test

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.221	3	.074	3.449	.036
Within Groups	.426	20	.021		
Total	.647	23			

Table 51. Multiple range test: mucus content of rats in ulcer treatment test

	Group	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	Hericium extract 200mg/kg	6	.2458	
	Ulcer Control Group	6	.2600	
	Hericium extract 400mg/kg	6	.2790	
	Omeprazole Group	6	.4813	
	Sig.		.050	

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

iii) Alcian blue

Table 52. Descriptives: alcian blue of rats in ulcer treatment test

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Ulcer Control Group	6	5.4173	.72640	.29655	4.6550	6.1796	4.39	6.47
Omeprazole Group	6	8.2568	2.13427	.87131	6.0170	10.4966	5.43	11.87
Hericium extract 200mg/kg	6	7.5778	1.77778	.72577	5.7121	9.4434	5.73	10.32
Hericium extract 400mg/kg	6	8.2938	.84154	.34356	7.4107	9.1770	7.06	9.65
Total	24	7.3864	1.83833	.37525	6.6102	8.1627	4.39	11.87

Table 53. ANOVA: alcian blue of rats in ulcer treatment test

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	32.970	3	10.990	4.911	.010
Within Groups	44.757	20	2.238		
Total	77.727	23			

Table 54. Multiple range tests: alcian blue of rats in ulcer treatment test

			Subset for alpha = 0.05	
	Group	**N**	1	2
Tukey HSD[a]	Ulcer Control Group	6	5.4173	
	Hericium extract 200mg/kg	6	7.5778	7.5778
	Omeprazole Group	6		8.2568
	Hericium extract 400mg/kg	6		8.2938
	Sig.		.090	.840

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

Appendix C: Raw data

a) Acute toxicity test

Body weight of rats

	Male									Female								
	Control group			Low dose group			High dose group			Control group			Low dose group			High dose group		
Replicate	1st day	7th day	14th day	1st day	7th day	14th day	1st day	7th day	14th day	1st day	7th day	14th day	1st day	7th day	14th day	1st day	7th day	14th day
1	279	305	313	242	263	270	245	282	283	205	219	212	210	205	214	168	174	180
2	280	299	305	210	233	230	245	284	300	168	168	182	196	199	204	203	205	210
3	230	255	269	230	250	255	280	319	325	188	178	185	222	225	226	212	222	219
4	256	298	311	223	254	251	190	208	212	180	190	199	257	253	250	206	196	206
5	190	230	241	240	261	270	176	203	204	164	167	175	225	233	240	202	211	221
6	250	285	293	211	225	225	240	279	289	213	216	220	188	194	210	208	208	208

Hematological parameters

HGB

	Male			Female		
Replicate	Control	Low dose	High dose	Control	Low dose	High dose
1	150	140	143	127	149	145
2	135	151	150	141	138	147
3	150	156	142	150	146	144
4	147	160	150	148	130	135
5	158	146	150	143	140	147
6	144	151	135	137	89	147

HCT

	Male			Female		
Replicate	Control	Low dose	High dose	Control	Low dose	High dose
1	0.47	0.45	0.47	0.42	0.5	0.48
2	0.44	0.49	0.5	0.46	0.45	0.48
3	0.49	0.5	0.47	0.5	0.48	0.48
4	0.48	0.51	0.49	0.48	0.43	0.44
5	0.52	0.47	0.49	0.49	0.46	0.49
6	0.48	0.49	0.45	0.43	0.43	0.48

RBC

	Male			Female		
Replicate	Control	Low dose	High dose	Control	Low dose	High dose
1	8.21	8.01	8.08	7	8.68	7.65
2	7.9	8.36	8.8	8.16	8.04	8.11
3	7.7	8.86	8.39	8.46	7.8	7.81
4	8.51	9.27	8.56	8.31	7.4	7.51
5	8.52	7.62	8.61	7.62	7.92	8.4
6	8.24	8.26	7.9	7.27	7.22	8.4

MCV

	Male			Female		
Replicate	Control	Low dose	High dose	Control	Low dose	High dose
1	57	56	58	60	57	63
2	55	59	56	57	56	59
3	64	57	56	59	61	62
4	56	55	57	57	59	59
5	61	62	57	64	58	58
6	58	59	57	60	60	57

MCH

	Male			Female		
Replicate	Control	Low dose	High dose	Control	Low dose	High dose
1	18.3	17.5	17.7	18.1	17.2	19
2	17.1	18.1	17	17.3	17.2	18.1
3	19.5	17.6	16.9	17.7	18.7	18.4
4	17.3	17.3	17.5	17.8	17.6	18
5	18.5	19.2	17.4	18.8	17.7	17.5
6	17.5	18.3	17.1	18.8	12.3	17.5

MCHC

	Male			Female		
Replicate	Control	Low dose	High dose	Control	Low dose	High dose
1	320	311	306	304	301	302
2	310	308	302	305	308	309
3	305	311	302	301	305	299
4	309	312	309	310	300	305
5	304	311	307	293	306	300
6	303	308	299	316	207	309

RDW

	Male			Female		
Replicate	Control	Low dose	High dose	Control	Low dose	High dose
1	14.1	15.6	15.6	15.9	14.9	14.9
2	14	14.2	16.3	14.7	13.9	14.1
3	16.4	14.1	17.2	13.3	13.8	13.4
4	15.5	16.4	15.7	16.8	13.7	13.5
5	16.8	13.4	14.2	15	13.8	14.7
6	17.4	14.9	17.1	14.6	13	13.4

WBC

	Male			Female		
Replicate	Control	Low dose	High dose	Control	Low dose	High dose
1	5.1	7.3	7.1	2.8	5.8	5.4
2	8.1	6	4.3	6.8	5.1	5.8
3	8.7	7.1	6.9	4.8	4.2	7.8
4	4.6	7.5	6.8	4.4	3	3.9
5	6.6	9.3	7.1	4.3	5.4	5.5
6	4.8	7.7	5.5	3.7	3	4.3

Platelet

	Male			Female		
Replicate	Control	Low dose	High dose	Control	Low dose	High dose
1	738	510	856	628	686	851
2	806	675	834	821	901	741
3	778	799	678	672	624	818
4	814	818	686	654	702	633
5	631	807	737	521	750	750
6	789	819	749	546	483	680

Renal function parameters

Sodium

	Male			Female		
Replicate	Control	Low dose	High dose	Control	Low dose	High dose
1	140	142	143	142	141	142
2	141	144	143	142	141	141
3	144	141	142	140	142	142
4	142	141	141	143	141	142
5	143	143	143	142	138	141
6	142	141	143	142	143	139

Potassium

	Male			Female		
Replicate	Control	Low dose	High dose	Control	Low dose	High dose
1	4.6	4.9	5.5	4.3	4.9	4.3
2	4.8	4.9	4.8	4.9	4.4	4.7
3	4.8	5.2	5.1	4.8	4.9	4.7
4	4.6	5.3	5.1	4.7	3.9	4.3
5	4.8	4.9	5	4.8	4.8	4.7
6	5	5.3	5	4.2	4.5	4.5

Chloride

	Male			Female		
Replicate	Control	Low dose	High dose	Control	Low dose	High dose
1	101	108	107	105	104	106
2	103	107	102	107	104	105
3	106	102	103	103	106	106
4	103	105	107	106	106	109
5	106	109	106	104	104	105
6	105	107	106	107	108	105

CO2

	Male			Female		
Replicate	Control	Low dose	High dose	Control	Low dose	High dose
1	26.3	20.9	24.4	23.8	24.1	24.1
2	25.1	23.1	26.9	21.9	23.7	23.3
3	25	27.8	26	24.1	22.2	21.8
4	25.1	22.2	19.9	22.9	21.5	18.6
5	22.3	19.2	22.5	24.6	22.6	22.3
6	25.1	22.4	23.8	22.7	23.6	21.6

Anion gap

	Male			Female		
Replicate	Control	Low dose	High dose	Control	Low dose	High dose
1	17	18	17	18	18	16
2	18	19	19	18	18	17
3	16	16	18	18	19	19
4	19	19	19	19	17	19
5	20	20	20	18	16	18
6	17	17	18	17	16	17

Urea

	Male			Female		
Replicate	Control	Low dose	High dose	Control	Low dose	High dose
1	4.6	7.5	5.7	7.2	7.1	11.3
2	4.9	7	5.2	8.6	7.8	8
3	4.4	4.4	6.2	6.8	8.3	7.3
4	5.5	5	6.1	8.7	8.2	8.9
5	6.8	6.4	5.4	8.1	8.4	7.5
6	5	6.9	5.2	8.2	8	6.8

Creatinine

	Male			Female		
Replicate	Control	Low dose	High dose	Control	Low dose	High dose
1	33	42	40	36	34	40
2	44	40	42	43	42	45
3	30	36	46	39	62	44
4	29	26	17	54	36	48
5	35	29	21	37	48	35
6	38	29	40	37	30	36

Liver function parameter

Total protein

	Male			Female		
Replicate	Control	Low dose	High dose	Control	Low dose	High dose
1	66	58	61	65	65	67
2	61	58	62	61	67	64
3	58	58	63	69	64	64
4	59	60	60	66	61	67
5	62	58	59	64	59	65
6	62	55	56	61	64	63

Albumin

	Male			Female		
Replicate	Control	Low dose	High dose	Control	Low dose	High dose
1	11	10	11	11	11	11
2	8	9	11	11	10	10
3	10	8	8	11	13	12
4	10	9	10	11	10	13
5	11	8	10	12	11	11
6	9	8	9	11	11	12

Globulin

	Male			Female		
Replicate	Control	Low dose	High dose	Control	Low dose	High dose
1	55	48	50	54	54	56
2	53	49	51	50	57	54
3	48	50	55	58	51	52
4	49	51	50	55	51	54
5	51	50	49	52	48	54
6	53	47	47	50	53	51

Total bilirubin

	Male			Female		
Replicate	Control	Low dose	High dose	Control	Low dose	High dose
1	2	2	2	2	2	2
2	2	2	2	2	2	2
3	2	1	2	2	2	2
4	2	2	2	2	2	2
5	2	2	2	2	2	2
6	3	2	2	2	2	2

Conjugated bilirubin

	Male			Female		
Replicate	Control	Low dose	High dose	Control	Low dose	High dose
1	1	1	1	1	1	1
2	1	1	1	1	1	1
3	1	1	1	1	1	1
4	1	1	1	1	1	1
5	1	1	1	1	1	1
6	1	1	1	1	1	1

ALP

	Male			Female		
Replicate	Control	Low dose	High dose	Control	Low dose	High dose
1	106	146	124	107	89	125
2	200	137	114	107	79	75
3	170	155	178	159	53	101
4	155	124	131	112	74	88
5	175	223	131	120	64	83
6	112	194	132	84	78	92

ALT

	Male			Female		
Replicate	Control	Low dose	High dose	Control	Low dose	High dose
1	58	48	44	43	49	47
2	55	47	50	40	46	44
3	53	49	50	37	37	47
4	52	48	44	45	37	44
5	48	50	42	51	31	36
6	45	46	49	43	38	49

AST

	Male			Female		
Replicate	Control	Low dose	High dose	Control	Low dose	High dose
1	155	182	190	177	146	190
2	201	169	159	177	202	201
3	173	163	208	142	197	160
4	159	193	165	168	153	187
5	160	197	153	193	150	180
6	181	187	180	171	185	179

GGT

Replicate	Male			Female		
	Control	Low dose	High dose	Control	Low dose	High dose
1	3	3	3	3	3	3
2	4	3	3	5	3	3
3	3	5	3	3	6	3
4	3	3	3	4	3	3
5	3	3	3	3	3	3
6	3	5	4	4	3	3

a) Anti-ulcerogenic study

	ulcer area						
	Normal group	Ulcer control group	Omeprazole group	Extract 50mg/kg	Extract 100mg/kg	Extract 200mg/kg	Extract 400mg/kg
1	0	864	252	792	504	496.8	237.6
2	0	842.4	280.8	640.8	633.6	396	172.8
3	0	936	316.8	669.6	720	367.2	208.8
4	0	756	360	712.8	583.2	525.6	273.6
5	0	993.6	223.2	741.6	604.8	518.4	280.8
6	0	972	280.8	806.4	568.8	417.6	266.4

	mucus content						
	Normal group	Ulcer control group	Omeprazole group	Extract 50mg/kg	Extract 100mg/kg	Extract 200mg/kg	Extract 400mg/kg
1	0.4135	0.7234	1.535	0.7566	0.8121	1.1464	1.3231
2	0.5908	0.8542	1.453	0.8592	0.9283	0.7588	1.0424
3	0.2057	0.6956	1.488	0.7984	0.8358	0.8571	1.2194
4	0.5554	0.7947	1.367	0.6982	0.6398	0.9478	1.1172
5	0.3385	0.8869	1.848	0.8983	0.8441	1.0493	1.1712
6	0.4586	0.8378	1.643	0.7659	0.8642	0.7999	1.2263

	Alcian blue						
	Normal group	Ulcer control group	Omeprazole group	Extract 50mg/kg	Extract 100mg/kg	Extract 200mg/kg	Extract 400mg/kg
1	3.207407	3.42963	5.355556	6.17037	10.54074	11.5037	13.57778
2	5.8	3.503704	9.133333	10.24444	9.874074	11.65185	11.13333
3	4.17	2.837037	7.725926	8.096296	12.76296	13.28148	8.392593
4	6.096296	3.503704	7.503704	9.948148	11.8	13.28148	12.83704
5	5.8	3.42963	4.911111	6.466667	11.8	10.02222	8.911111
6	4.985185	3.8	5.725926	7.651852	10.39259	14.02222	8.985185

	MDA						
	Normal group	Ulcer control group	Omeprazole group	Extract 50mg/kg	Extract 100mg/kg	Extract 200mg/kg	Extract 400mg/kg
1	4.52657	9.067633	5.299517	7.183575	6.555556	5.7343	5.10628
2	4.478261	8.729469	5.637681	7.47343	7.038647	6.217391	5.299517
3	4.285024	8.874396	5.396135	7.714976	6.89372	6.120773	5.347826

	SOD						
	Normal group	Ulcer control group	Omeprazole group	Extract 50mg/kg	Extract 100mg/kg	Extract 200mg/kg	Extract 400mg/kg
1	8.260235	1.642295	5.369144	2.011252	5.029921	6.310204	5.691669
2	9.328042	2.198369	5.976743	1.904177	4.080923	5.536923	5.470084
3	8.860137	1.826241	5.964269	2.394101	3.950272	6.869823	4.78879

	CAT						
	Normal group	Ulcer control group	Omeprazole group	Extract 50mg/kg	Extract 100mg/kg	Extract 200mg/kg	Extract 400mg/kg
1	166.6071982	106.9345816	62.31956884	37.89692563	83.11101505	146.2662149	130.218007
2	203.011208	129.295061	75.3200287	45.8620604	117.263649	123.838596	133.489721
3	168.113376	100.079893	67.9043941	37.5977055	88.2662547	137.71901	114.558563

b) Ulcer healing

	ulcer area				
	Normal control group	Ulcer control group	Omeprazole group	Extract 200mg/kg	Extract 400mg/kg
1	1411.2	468	230.4	108	0
2	1310.4	511.2	324	64.8	0
3	1238.4	403.2	244.8	57.6	0
4	1706.4	590.4	158.4	86.4	0
5	1591.2	576	252	43.2	0
6	1360.8	540	288	79.2	0

	mucus content				
	Normal control group	Ulcer control group	Omeprazole group	Extract 200mg/kg	Extract 400mg/kg
1	0.5688	0.2532	0.9156	0.2438	0.3245
2	0.5796	0.1475	0.5166	0.2251	0.2113
3	0.5416	0.1053	0.4795	0.2543	0.3378
4	0.7504	0.3066	0.248	0.3364	0.134
5	0.3833	0.3517	0.283	0.1005	0.2881
6	0.5266	0.3959	0.4452	0.3146	0.3784

	Gastric mucus barrier				
	Normal control group	Ulcer control group	Omeprazole group	Extract 200mg/kg	Extract 400mg/kg
1	16.39259	6.466667	9.059259	10.31852	8.17037
2	14.83704	5.948148	11.87407	6.17037	9.651852
3	11.13333	4.392593	7.355556	9.059259	8.096296
4	10.0963	5.42963	5.42963	5.725926	7.059259
5	10.17037	4.985185	7.948148	6.762963	8.17037
6	12.24444	5.281481	7.874074	7.42963	8.614815